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OF

EXPERIMENTAL PATHOLOGY

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

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> “Si vous ne pensez pas, créez de nouveaux mots.”
>
> Voltaire. *Satires.*

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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

A COMPLEMENT-FIXATION TEST IN COCCIDIOSIS OF THE RABBIT.

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Forwarded for publication October 16th, 1922.

RECENTLY an epidemic of coccidiosis (*Eimeria stiedæ*, Lindemann, 1865) occurred amongst the rabbits bred for experimental purposes in the Institute, and as a result about forty young rabbits succumbed. Many others were affected but are recovering. As a means of sorting out the affected from those non-affected, the complement-fixation test suggested itself. On searching the literature, I find that Kuczynski (1921) discovered that the serum of rabbits spontaneously or intentionally infected with coccidia gave a positive Wassermann reaction. He believes that the fact, recorded by Friedemann and by Wendtlandt, that rabbit serum often gives a positive reaction, is to be explained by the frequency of coccidiosis amongst these animals.

TECHNIQUE.

Antigens.—As coccidiosis affects mainly the liver and the mucous membrane of the upper part of the small intestine, the antigens used were prepared by extracting the infected livers with absolute alcohol, with carbolised normal saline solution, and with normal saline (0·85 per cent.) solution.

(1) Alcoholic extract: 5 grm. heavily infected liver were ground in a mortar with an equal quantity of kieselgühr, and 40 c.c. of absolute alcohol added. The mixture was put in a stoppered flask and incubated at 37° C. for two days, being shaken several times a day. It was filtered and the filtrate was kept on ice till required. A clear yellow fluid was obtained.

(2) Carbol-saline extract: This was prepared as in No. 1 antigen, using, however, normal saline (to which 0·5 per cent. of carbolic acid had been added) as a solvent instead of alcohol. A fairly clear yellow filtrate was obtained.

(3) Normal saline extract: 5 grm. infected liver were rubbed up with kieselgühr and 40 c.c. of normal (0·85 per cent.) saline in a mortar, and the mixture was placed on ice in a closed flask and shaken thoroughly several times a day. It was filtered before use, but a very turbid filtrate resulted.

Hæmolytic systems.—Hæmolysins prepared from rabbits against sheep and ox-corpuscles respectively were used with the corresponding cells; the cells received maximum sensitisation with 4 M.H.D. of hæmolysin.

Complement.—Fresh serum from healthy male guinea-pigs was used as complement. This was titrated as a preliminary with the hæmolytic system, and used in a dilution so that unit volume (2 drops) contained 3 M.H.D.

The dropping method was used throughout, 2 drops (unit volume) being 0·2 c.c. with the pipettes used.

Preliminary tests were carried out to determine the inhibition of complement by the antigen alone. Two drops of antigen in diluted series were put up with 4 drops of saline solution and 2 drops of diluted complement (3 M.H.D.). After incubation for one hour at 37° C., 2 drops of the sensitised cells were added, and the degree of hæmolysis read at the end of another hour in the incubator at 37° C. Table I shows the results obtained in one experiment.

TABLE I.

Hæmolytic system.	Antigen.	Dilution.							
		1	1/2	1/3	1/6	1/9	1/12	1/24	1/36
Sheep—anti-sheep	alcoholic	+ + +	+ + +	+	—	—	—	—	—
	carbol-saline	—	—	—	—	—	—	—	—
	normal saline	+	—	—	—	—	—	—	—
Ox—anti-ox	alcoholic	+ + +	+ + +	—	—	—	—	—	—
	carbol-saline	—	—	—	—	—	—	—	—
	normal saline	+	—	—	—	—	—	—	—

— = complete hæmolysis, + = almost complete hæmolysis, + + = half cells hæmolysed, + + + = no hæmolysis.

For the test then the alcoholic extract was used in a dilution of 1 to 9 parts of normal saline solution, and the carbol-saline and normal saline extracts in dilutions of 1 to 2 parts of normal saline. The test was set up with four tubes for each serum, which was diluted 1 in 5 with saline and inactivated for 20 minutes in a water-bath at 56° C. The first three tubes contained serum, antigen, and 3, 4½, and 6 M.H.D. of complement respectively, while the fourth tube, the control, contained serum and 3 M.H.D. of complement. Each tube was made up to 0·8 c.cm. with saline. Controls of complement and complement with antigen and with double-strength antigen were set up in each set. The sets were put in the incubator at 37° C. for one hour, then 0·2 c.c. of sensitised red cells added, and the incubation continued for one hour longer.

TABLE II.

Extract . . .	Alcoholic 1/10.								Carbol sal. 1/3.								Normal sal. 1/3.							
Hæmolytic system .	Sheep.				Ox.				Sheep.				Ox.				Sheep.				Ox.			
Complement M.H.D.	3.	4½.	6.	3.	3.	4½.	6.	3.	3.	4½.	6.	3.	3.	4½.	6.	3.	3.	4½.	6.	3.	3.	4½.	6.	3.
Rabbit, infected . .	−	−	−	−	−	−	−	−	+ + +	+ +	−	−	+ +	−	−	−	+ + +	+ +	−	−	+ + +	+ + +	+ +	−
,, ,, . .	+	−	−	−	−	−	−	−	+ + +	+ + +	+	−	+ + +	+	−	−	+ + +	+ + +	+ +	−	+ + +	+ + +	+ + +	−
,, ,, . .	−	−	−	−	−	−	−	−	+ + +	+ +	−	−	−	−	−	−	+ + +	+ + +	+ + +	−	+ + +	+ +	−	−
,, ,, . .	+	−	−	−	−	−	−	−	+ + +	+ + +	−	−	+ + +	−	−	−	+ + +	+ + +	−	−	+ + +	+ +	−	−
,, ,, . .	+	−	−	−	−	−	−	−	+ +	−	−	−	−	−	−	−	+ + +	+ + +	−	−	+ + +	−	−	−
,, recovering . .	−	−	−	−	−	−	−	−	+ + +	−	−	−	−	−	−	−	+ + +	+ + +	−	−	+ + +	+ +	−	−
,, normal . .	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+ +	−	−	−
,, ,, . .	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
,, old scarred liver .	+	−	−	−	−	−	−	−	+ + +	+ + +	+ +	−	+ + +	+ +	−	−	+ + +	+ + +	+ + +	−	+ + +	+ + +	+ + +	−
Guinea-pig	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Human, positive Wassermann reaction . . .	+ + +	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+ + +	+	−	−	+ + +	−	−	−
Human, positive hydatid reaction	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Control; complement alone, do. + antigen, do. + double strength antigen . .	−	−	−		−	−	−		−	−	+		−	−	−		−	−	+ +		−	−	−	

The sera of at least two rabbits known to be healthy were put up at each test.

A typical protocol obtained is shown in Table II (p. 3).

RESULTS.

Sera from three rabbits proved to be normal and from twenty-nine other rabbits known or suspected to be infected were examined, and the results of the series of tests are summarised in Table III.

TABLE III.

Antigen . .		Alcoholic extract.		Carbol-saline.		Normal saline.	
System . .		Sheep.	Ox.	Sheep.	Ox.	Sheep.	Ox.
Normal .	No. tested . .	3	3	3	3	3	3
	Strong reaction .	0	0	0	0	0	0
	Reaction . .	0	0	0	0	0	1
	No reaction. .	3	3	3	3	3	2
Infected .	No. tested . .	17	17	29	29	29	29
	Strong reaction .	0	0	9	12	17	17
	Reaction . .	2	2	5	8	9	11
	No reaction. .	15	15	15	9	3	1

The alcoholic extract, which fixed complement in the presence of human serum giving the Wassermann reaction, proved a poor antigen even in dilutions of 1 in 6, and in stronger amounts tended to fix complement itself. The carbol-saline extract of the infected livers had good antigenic properties, and showed no anticomplemetary tendency. The normal saline extract acted as a good antigen, but tended to become anticomplementary on keeping.

Note.—I have to acknowledge the great assistance of Miss F. E. Williams in carrying out the technical part of the work.

REFERENCE.

KUCZYNSKI, M. H.—(1921) *Berlin. klin. Wchnschr.*, **58**, 125.

THE INFLUENCE OF VITAMIN A ON THE BLOOD-PLATELETS OF THE RAT.

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AND

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Received for publication January 3rd, 1923.

In a recent communication Cramer, Drew and Mottram (1922) describe a series of experiments in which a specific lesion, not previously observed, is manifested in rats kept on a diet free from vitamin A. This lesion is characterised by a reduction in the number of blood-platelets which was found to vary with the intensity of the dietetic deficiency. One need hardly enlarge on the physiological significance of such a phenomenon if it could be confirmed. Our interest in this work was particularly stimulated by the possibility of utilising this observation in the quantitative estimation of vitamin A. It has been already pointed out by Zilva and Miura (1921) that in the case of a vitamin A deficiency there is no clear-cut symptom as in the case of scurvy and polyneuritis, which can be utilised as an " end-point " in the quantitative estimation of the vitamin. Resumption of growth by rats fed on a vitamin A deficient diet under specified conditions mainly devised to eliminate vigorously growing animals in the initial stages of the deficiency was, therefore, suggested by them as a standard. Good results have been obtained by this method. It is recognised, however, that the employment as a standard of a more decisive and specific lesion, such as a progressive thrombopœnia, would greatly enhance the quantitative method described by Zilva and Miura. According to Cramer, Drew and Mottram, a diminution in the number of platelets can be recorded in vitamin A deficient rats independent of their growth propensities, and this change becomes markedly manifest before the infective conditions make their appearance. The average of the platelet counts obtained by these workers in normal rats is 851,000 per c.mm., whilst the average of their counts in vitamin A deficient rats is 378,000, the lowest platelet recorded by them in this latter category of rats being 154,000 per c.mm.

Our preliminary observations were made with the object of corroborating these findings of Cramer, Drew and Mottram. Platelet counts were made on vitamin A deficient rats and compared with counts in normal rats of the same age. No marked divergence between the platelet counts in these two categories of rats was observed. This failure urged us to repeat Cramer, Drew and Mottram's work in detail. These authors find that the platelet count falls not only in rats which show little or no initial growth immediately after being placed on the deficient diet, which animals they designate for the sake of convenience as "acute," but also in rats which show a marked tendency to grow for some time before the deficiency is demonstrated by their weight curve and which are described as "chronic." On administering the fat-soluble factor the number of platelets increased and eventually reached the normal count. We therefore investigated the three categories of animals.

The deficient diet of our animals consisted of the following ingredients:

Inactivated casein	20 parts.
Starch	50 ,,
Salt mixture	5 ,,
Marmite	5 ,,
Decitrated lemon-juice . . .	5 ,,
Hardened cotton-seed oil . . .	15 ,,

The casein was inactivated by being heated for 48 hours in shallow trays and frequently turned over at a temperature of 120° to 130° F. The salt mixture employed was of the same composition as that used by McCollum and his colleagues. This diet was found from several years' experience to be very deficient in vitamin A, and is used extensively for the quantitative estimation of this vitamin.

TECHNIQUE OF THE PLATELET COUNT.

The method employed during this investigation for the enumeration of the platelets is the same in principle as the one described in a previous communication (Bedson, 1921). The ratio of red cells to platelets is first obtained in a preparation of citrated blood mounted between slide and cover-glass, and the absolute red count is then made by means of the Thoma-Zeiss pipette and counting chamber. By means of these data the absolute platelet count is calculated. In view of the fact, however, that some parts of the technique had to be modified to render it applicable to the rat, it would be perhaps advisable to describe the method in detail.

Method for obtaining the blood for the red-cell-platelet ratio.—The blood for this estimation was obtained by one of two methods:

(1) By cardiac puncture.

(2) By cutting the tail whilst immersed in an anti-coagulant.

In carrying out the first procedure (cardiac puncture) the rat is anæsthetised with ether and the chest-wall shaved. A small all-glass syringe fitted with a comparatively wide-bore needle is used. This is thoroughly washed out with normal saline containing 2 per cent. of sodium citrate and then 0·5 c.c. of the same citrate-saline solution is drawn up into the syringe and forced to the tip of the needle. The point at which to insert the needle is readily determined by palpation. The needle is plunged into the heart, about 0·05 c.c. of blood drawn up and the needle withdrawn. The blood is then rapidly mixed with the citrate by rotating the syringe, the needle removed and the citrated blood expelled into a paraffined watch-glass. In order to obtain a satisfactory preparation by this method the needle must enter the heart at the first attempt, and as soon as the blood has entered the syringe it must be thoroughly mixed with the diluent with as little delay as possible. Should the first attempt at cardiac puncture not be successful, then the needle must be withdrawn and thoroughly washed out with the citrate solution.

The second method is the one described by Cramer, Drew and Mottram. It consists of cutting the tail whilst immersed in the diluent (2 per cent. citrate in saline). As soon as the blood is flowing freely, the tail is transferred to a second quantity of citrate saline and allowed to remain there until the requisite amount of blood has been obtained. These operations are carried out with the rat deeply etherised. This is essential if a free flow of blood is to be obtained, as Cramer, Drew and Mottram have pointed out.

In our hands the first of these two methods, viz. cardiac puncture, has proved the most satisfactory. The risk of any action on the platelet by tissue juices is reduced to a minimum, and there is not the difficulty of getting the animal to bleed, which is sometimes the case with the "tail" method. However, cardiac puncture has its limitations. It is very difficult to perform on a young rat of only 50 grm. weight, and it is not applicable to those cases where a series of counts is to be made on an individual rat, owing to the risk of killing the animal. This being so, we have made use of this method only where isolated platelet counts were being made, and when it was desired to make a series of observations on individual rats the blood has been obtained from the tail.

The red-cell-platelet ratio estimation.—The blood, obtained by one or other of the methods just described, is thoroughly mixed, and a suitable quantity transferred to a slide by means of a paraffined pipette or platinum loop, covered immediately with a cover-slip and ringed with vaseline. The quantity of blood taken should be such as to spread out evenly between the slide and cover-slip without causing the latter to float. The number of platelets and red cells in a series of fields of this preparation is then counted. The size of field selected should not be too large, otherwise the number of red cells to be counted becomes too great. Needless to say, also, the preparations should show no clumping of platelets, and the dilution of the blood should be such as to give well-isolated elements. However carefully the preparation be made it will be found that the distribution of the platelets is not absolutely regular. In view of this fact *a series of fields is counted right across the preparation from one side to the other.* If sufficient platelets have not been counted in traversing the preparation once, then it must be moved either up or down and traversed again in the opposite direction. *At least 100 platelets must be counted.* The counting can be done either with a 4 mm. objective and fairly high ocular or with the oil immersion. The illumination can be either by means of a dark-ground condenser, or by an ordinary condenser with the iris diaphragm closed sufficiently as to give clear definition. The actual counts which form the subject of this communication were made either with objective 6 and ocular 4 (Koristka), or with objective $\frac{1}{15}''$ semi-apochromat. and compensating ocular 8 (Koristka). An Abbe condenser (N.A. 1·3) was used in both instances. In many cases the same preparation has been counted both with the dry lens and the oil immersion and similar counts obtained. By counting the preparation right across from side to side and by always moving the preparation on with the eye removed from the microscope—thus avoiding any selection of field—the personal factor has been reduced to a minimum.

EXPERIMENTAL FINDINGS.

Our first observations, as has been pointed out, consisted of isolated platelet counts on normal and vitamin A deficient rats. These rats were all of approximately the same age—a point of importance when one remembers how rapidly the blood picture changes in the first few months in the life of these animals. The figures obtained are tabulated below (Tables I and II). The blood for the red cell-platelet ratio determination was obtained in every instance from the heart. The counting was done with objective 6 and ocular 4 (Koristka), and in certain instances the counts so obtained were checked by recounting the preparation with objective $\frac{1}{15}$ inch and compensating ocular 8 (Koristka). As stated already, no appreciable difference was found between the counts made with these two different magnifications.

TABLE I.—*Platelet Counts obtained in 7 Normal Rats.*

Red cell.	R.P. ratio.	Platelets.
6,896,000	6·3	1,094,000.
7,776,000	6·8	1,143,000.
7,840,000	7·0	1,120,000.
8,040,000	5·7	1,585,000.
8,448,000	7·2	1,173,000.
8,080,000	7·5	1,077,000.
7,520,000	5·9	1,270,000.
Average of above counts.		
7,800,000		1,208,857.

It will be observed that the figures obtained for the platelets in these seven rats vary between extremes of 1,585,000 and 1,077,000 per c.mm. The average count, 1,208,857 is considerably higher than what is found in the guinea-pig and rabbit.

TABLE II.—*Platelet Counts in 9 Vitamin A Deficient Rats.*

Red cells.	R.P. ratio.	Platelets.	Remarks.
7,800,000	5·4	1,440,000	Ceased growing. No eye change.
8,360,000	6·8	1,229,000	,, ,,
7,736,000	6·5	1,190,000	Well-developed keratomalacia.
7,120,000	8·7	818,000	Moderate degree of ,,
7,568,000	6·5	1,164,000	Well-developed ,,
8,336,000	9·28	898,000	,, ,,
7,760,000	8·6	902,000	,, ,,
7,568,000	7·7	982,000	,, ,,
8,272,000	7·8	1,060,000	,, ,,

	Average of above counts.
7,946,666	1,075,888
	Average of counts in those rats with keratomalacia.
7,908,555	1,002,000

The platelet counts in these nine rats are, generally speaking, slightly lower than those obtained in normal rats, and this is certainly more evident in some of those animals which had developed definite eye change. The difference between the average of these nine counts and the seven normal counts is only 122,969, or if we exclude the two deficient rats which had not developed keratomalacia it is 206,857. Although the difference is probably more than could be accounted for by experimental error, it by no means represents the very considerable fall in the platelet count experienced by Cramer, Drew and Mottram. If the withholding of vitamin A from rats had as its specific result a considerable diminution in the number of platelets, then one would have expected the above counts, representing as they do the two extremes of the picture, to have revealed a considerable difference between normal and deficient rats. It was recognised, however, that a more satisfactory procedure would be to make a series of platelet counts on rats both before and during the course of a vitamin A deficiency. This was carried out on six rats, and the findings both as regards the weight and the platelets have been charted as curves in Fig. 1 and 2. In making these counts the blood has been obtained from the tail, and the counting carried out with objective Koristka $\frac{1}{16}$ inch semi-apochromat. and compensating ocular 8.

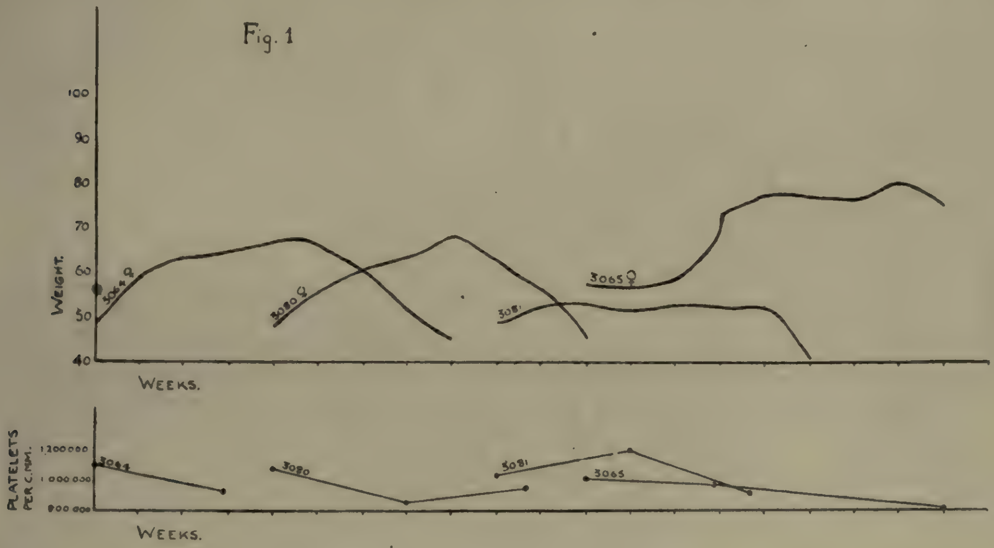
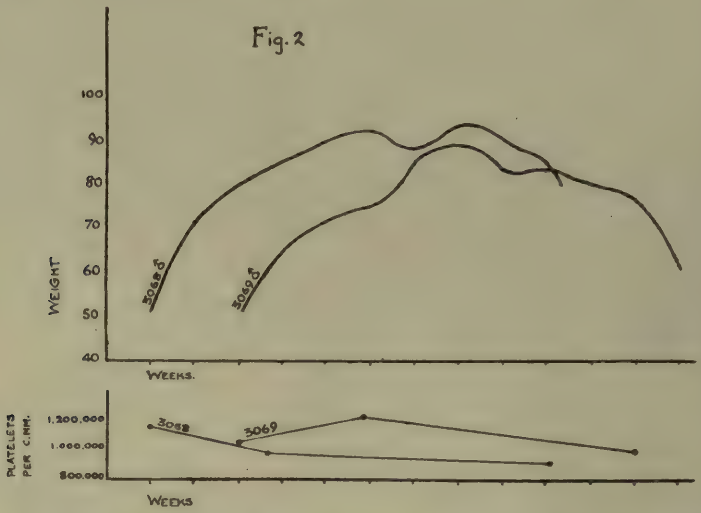


Fig. 1

Fig. 1 represents the weight curve of animals which can be classified as "acute," whilst in Fig. 2 the growth of rats belonging to the "chronic" category is graphically portrayed. It is to be pointed out that the "chronic" animals did not attain such a high weight as their prototypes in Cramer, Drew and Mottram's experiments. This is due to the fact that they were started on the deficient diet at a lower weight. Possibly they may also not have had the same reserve of the vitamin. Their rate of growth, however, was distinctly more intense than that of the "acute" group.

An examination of the data recorded in Figs. 1 and 2 reveals the fact that here again, though vitamin A deficiency is accompanied by a rather low platelet count, the fall recorded is very small.

Fig. 2



In order to bring out this point more clearly the actual platelet counts, together with the percentage fall in each case, are given below (Table III).

TABLE III.

Rat.	Platelet count before commencing experiments.	Final platelet count.	Difference between two counts.	Percentage change.	Time on −A diet between two counts.
3064	1,126,000	924,000	−202,000	17·9	3 weeks.
3065	1,009,000	807,000	−202,000	20·0	8 ,,
3068	1,143,000	899,000	−244,000	21·3	9 ,,
3069	1,054,000	996,000	−58,000	5·5	9 ,,
3080	1,090,000	953,000	−37,000	3·3	5·75 ,,
3081	1,031,000	847,000	−184,000	17·8	5·75 ,,

It will be seen that the greatest fall recorded, that in rat No. 3068, is only one of 244,000, or a 21·3 per cent. change, and that in three other rats, Nos. 3064, 3065 and 3081, a fall of somewhat similar degree has taken place. In the remaining two rats there is little change, though in these, again, the change is on the negative side. In order to adjudge small changes in the platelet count, such as we are dealing with, some idea of the experimental error in the technique employed is necessary. Three counts were, therefore, made on a normal rat at intervals of an hour, using the same technique as had been employed for the above estimations. The following figures were obtained (Table IV):

TABLE IV.

Count.	Platelets per c.mm.
1	1,195,000
2	1,128,000
3	1,321,000

The mean of these three counts is 1,214,000, and the average deviation from this mean 70,666, or 5·8 per cent. It is, of course, recognised that the number of observations is really too small to draw any satisfactory conclusion therefrom. The calculated error probably represents too low an estimate, especially when one remembers that this platelet count is subject to the error of the red cell count as well. The error is most probably in the neighbourhood of 10 per cent., not less, and if we accept this estimate then four out of the six counts recorded in Table III show a fall which lies beyond the limits of experimental error, while the remaining two are certainly within these limits. However, all the changes recorded in the six deficient rats are in the same direction—a diminution—and the probability is that this represents an actual change.

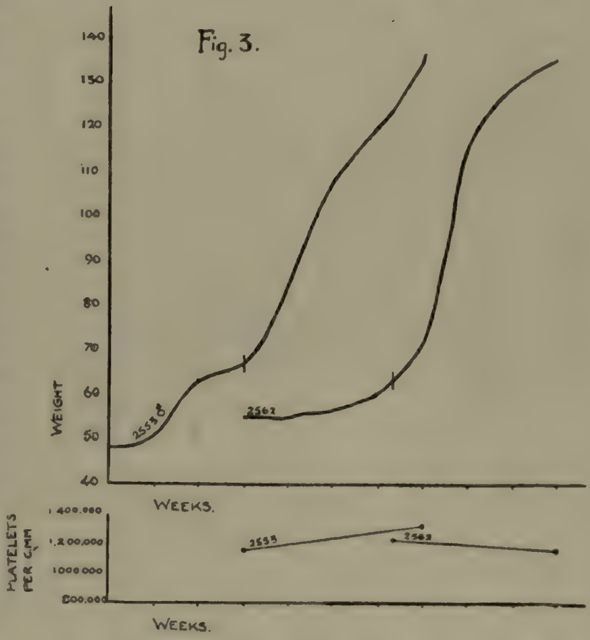


Fig. 3.

Finally, two vitamin A deficient rats were counted and then cod-liver oil was added to their diet. At the end of four weeks, when these two rats had gained very considerably in weight, their platelets were again counted. The findings, recorded graphically in Fig. 3, speak for themselves.

DISCUSSION.

A consideration of our findings reveals the fact that young growing rats when deprived of vitamin A show a slightly lower platelet count than do normal rats. In those of our experiments where consecutive counts were made, the general tendency of the platelet count is in the direction of a diminution, but even here, accepting the experimental error as 10 per cent., only four out of the six rats show a fall beyond this limit. The greatest diminution recorded by us was 244,000—a fall of 21·3 per cent. This is considerably less than the diminution in the number of platelets observed by Cramer, Drew and Mottram, and in view of our findings we feel unable to associate ourselves with their opinion that a "thrombopœnia" is a specific lesion of a vitamin A deficiency. We are not at all convinced that this minimal change in the platelet count could not be produced by other dietetic deficiencies, and the few observations on this point brought forward by Cramer, Drew and Mottram do not seem to us of very great value. In the first place they consist of isolated observations only, and secondly, no mention is made of the age of the rats which were examined after being placed on diets deficient in vitamin B. This question of age seems to us of great importance. As is well known, the blood picture in the rat undergoes very considerable changes in the first three months of life. These changes concern more particularly the number of formed elements per unit volume—that is to say, that in the young growing rat the rate of production of formed elements outstrips the production of plasma, with a consequent increasing concentration of formed elements. It is therefore possible that the low platelet counts observed in young growing rats deprived of vitamin A are merely due to the fact that platelet production is slowed rather more than the production of the other elements of the blood, and that a similar change would not be observed in mature rats fed on the same vitamin A free diet. In order to prove this point it would be necessary to work out carefully the platelet content in the blood of rats at frequent intervals during the first three months of life, and also to examine the blood of mature rats deprived of vitamin A. This has not been done. We can offer no explanation of the discrepancy between our findings and those of Cramer, Drew and Mottram.

CONCLUSIONS.

(1) Young growing rats fed on a vitamin A deficient diet show a lower platelet count than do normal rats of the same age.

(2) The greatest diminution in the platelet count observed by us in rats deprived of vitamin A was 244,000, representing a 21·3 per cent. reduction.

(3) We do not consider that this minimal change in the platelet count of vitamin A deficient rats can be regarded in the light of a specific lesion.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

REFERENCES.

Bedson, S. P.—(1921) "Blood-Platelet Anti-serum, its Specificity and *Rôle* in the Experimental Production of Purpura," *J. Path. & Bacteriol.*, **25**, 94.

Cramer, W., Drew, A. H., and Mottram.—(1922) "On Blood-Platelets; their Behaviour in 'Vitamin A' Deficiency and after 'Radiation,' and their Relation to Bacterial Infections," *Proc. R. Soc.*, B, **93**, 449.

Zilva, S. S., and Miura, M.—(1921) "The Quantitative Estimation of the Fat-Soluble Factor," *Biochem. J.*, **15**, 654.

VARIATIONS IN SOME OF THE URINARY CONSTITUENTS AND THE ALVEOLAR CARBON DIOXIDE TENSION IN RELATION TO MEALS.*

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THIS communication, which forms part of a series of investigations into the means adopted by the body to keep the reaction of the blood constant during meals, deals with the urinary factors. As much work has already been done on this subject, it will be as well to summarise the results obtained by previous workers.

PREVIOUS OBSERVATIONS ON THE REACTION OF URINE.

Variations in the urinary acidity have been known for a very long time, but Bence Jones was certainly the first to give any definite information on the subject. His original articles could not be consulted, yet it appears that he described an alkaline tide following meals, ascribing this to the secretion of HCl, thus leaving the blood with a tendency to alkalosis. The excretion of alkali into the urine would tend to correct this. Later Quincke (1884) stated that the changes were not constant, and that the most marked variations were diurnal in origin, being completely dissociated from the ingestion of food. Since these times a great deal of work has been done, some observers (Campbell, 1920) stating that an alkaline tide following meals occurs in practically all cases, whilst on the other hand, it has been held (Leathes, 1919) that this tide either does not exist or appears very rarely. In the opinion of the writer previous workers have run very great risks in the selection of their subjects, since in none of the literature is there any mention of how these investigators knew that their subjects secreted HCl. Bennett and Ryle (1921), in their 100 normal gastric analyses, encountered 4 per cent. of complete achlorhydrics, and the writer, in the course of a routine examination of patients and students in conjunction with Dr. Bennett, has been surprised with the large number of men, to all intents and purposes normal, who showed this condition.

Achlorhydria is so common that it must be excluded by a test-meal before a subject can be accepted for any form of gastric research. Everyone examined for urinary changes had undergone the continuous test-meal, and was thereby proved to have a normal secretion of acid.

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* A Report to the Medical Research Council.

METHODS.

The effect of the breakfast and lunch was investigated. In the case of the former, the subject was instructed to pass all his urine on retiring for the night, and to note the time. On rising in the morning the bladder was emptied and the time was again noted. After shaving, etc., he passed the urine secreted since the last specimen, and both of these were put into separate bottles, labelled, and their time intervals very carefully noted. Breakfast was then taken, the time again being carefully noted. He then came up to the laboratory with his two specimens, and passed urine into separate vessels at stated intervals of time. Some of these observations were prolonged for ten hours, others for shorter periods. In the case of the mid-day meal, specimens were collected before and after the meal as described above. Wherever possible, simultaneous alveolar CO_2 tensions were determined; but unfortunately these could not be obtained in the case of breakfast observations. The meals were made as standard as possible, consisting of, for breakfast, half a pint of tea, bacon and eggs, bread and butter; for lunch, meat, potatoes, bread, cheese, and half a pint of water were taken. During the observations the subjects were at rest in the laboratory, reading.

ANALYSIS OF SPECIMENS.

The volume of each specimen was recorded, and the "titrable acidity" and "ammonia" values were determined in the following way:

To 5 c.c. of urine 20 c.c. of boiled distilled water was added, followed by 0·75 c.c. of cresol red as an indicator. Neutral potassium oxalate (10 gm.) was added in order to precipitate those salts which tend to obscure the end-point. A standard solution of pH 7·4 was prepared by means of Sörensen's buffer phosphate solutions, and 0·75 c.c. of the cresol red solution added. The resulting solution was of a brownish colour, and was placed in a comparator. The urine was titrated to the colour standard by the addition of N/100 acid or alkali, according to whether it was acid or alkaline.

All comparisons were made in the comparator.

The titrable acidity having been determined, the ammonia content was arrived at by Malfatti's (1908) formalin method. To the solution obtained above 10 c.c. of 20 per cent. formalin was added, the formalin having been neutralised in the manner described for the estimation of titrable acidity. Owing to a peculiar obscuring action of the formalin on the indicator, Folin's (1919) air current method had to be used in a number of cases. Analyses were carried out on the day of the experiment whenever possible, and, in the case of delay, the urine was preserved by the addition of 1 c.c. of chloroform.

CALCULATION OF THE RESULTS.

It is at once obvious that the figures obtained by expressing the acidity and ammonia content as percentage c.c. N/100 alkali are not comparable in the various specimens. No account is taken of the varying intervals of time, which, owing to the nature of the experiments, are not always regular, nor of the amount of urine passed in that time, which naturally varies. Perhaps the fairest way to express the results is by excretion of acid or ammonia per hour, which embraces both the above factors. This value is arrived at as follows:

Let X c.c. be the quantity passed in Y minutes.

Let a be the number of c.c. of N/100 acid required to neutralise 100 c.c. of urine, *i. e.* the percentage acidity.

Then the amount of urine passed per hour will be $\frac{60\ X}{Y}$ c.c.

Since the percentage acidity is a c.c. N/100, the acid excretion per hour will be $\frac{a}{100} \times \frac{60\ X}{Y}$ c.c.

The ammonia excretion per hour is arrived at in the same manner.

The A and N value, *i. e.* titrable acidity per hour *plus* ammonia as c.c. $\frac{N}{100}$ alkali per hour, is regarded by Henderson as a useful index of the "acid" or "alkaline" tendencies of the body, and these, together with the CO_2 tension readings, will be found in the following tables:

As the results seem rather irregular, it was thought that the actual pH of the urine might give some valuable information. This was determined in some of the later cases by means of indicators and is appended to the respective tables.

RESULTS OF PRESENT OBSERVATIONS*: THE URINE IN RELATION TO MEALS.

Stated briefly, the present observations show that an alkaline tide follows the first meal of the day. After this period of decreased acid excretion, which lasts usually about one to two hours, an acid tide appears, lasting for two to three hours. Now as lunch is usually taken about four hours after breakfast, any urinary changes which would follow this meal are obscured by the acid tide of breakfast; hence the alkaline tide following this meal is either absent, or shows itself as a very slight drop in the A and N value.

(1) *The breakfast effect.*—Case 1, Table I. The alveolar carbon dioxide tension follows its usual course, the rise being 8 mm.—rather greater than usual. After the meal the urine becomes immediately alkaline, and there is a steady drop for 2½ hours. The hourly excretion of acid and NH_3 show a very steady drop, and then a rise, reaching a maximum 3½ hours after the meal. Six normal men were examined in this manner, and the results were the same in each case. One only is published (Case 1). It will be noted that the rise in the A + N value occurs at the time when lunch is usually taken, consequently any effect that this meal would have on the urine would be obscured by the breakfast effect. To investigate this point a series of all-day observations were made. Six experiments of this type were made, and two are given in the table (Cases 3 and 4). The breakfast effect is as already described, but the lunch produces either no effect at all, or at the most a very temporary drop in the A + N value. Hence, if the lunch effect be investigated, very misleading results are obtained, as can be seen from Case 5, one of a series of seven.

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* Owing to the expense of publishing large tables only typical results are reproduced for each group of experiments. It is hoped to publish the whole series of tables in a later and fuller report. Part of the cost of publishing this paper is being borne by the Medical Research Council, to whom the writer wishes to express his thanks.

TABLE I.

	Minutes.	Alv CO_2 mm.Hg.	Volume.		Acidity (Ph 7·4) c.c. N/100 alkali.			NH_3 c.c. N/100.			A+N per hour.	pH.
			c.c. total.	c.c. per hour.	Per cent.	In whole amount.	Per hour.	Per cent.	In whole amount.	Per hour.		
CASE 1.												
7.0–9.0 . . .	120	38·76	50	25	290	145	72·5	395	197·5	98·75	171·3	5
Meal 9–9.15												
9.0–9.35 . . .	35	41·04	35	60	−28	−9·8	−16·8	123	43·05	91	74·2	6·8
9.35–10.5 . . .	30	41·8	50	100	−19	−9·5	−19	83	41·5	83	64	8·2
10.5–10.35 . . .	30	44·08	90	180	−20	−18	−36	27	24·3	48·6	12·6	7·5
10.35–11.5 . . .	30	46·36	39	78	−65	−25·4	−50·8	45	17·5	35	−15·8	9·5
11.5–11.35 . . .	30	41·80	62	124	−18	−12·2	−24·4	66	41	82	59·6	7·9
11.35–12.5 . . .	30	40·28	43	86	−0·2	−0·1	−0·2	129	55·5	111	109·8	7·5
12.5–12.30 . . .	25	38	36	86	21	7·5	18	189	68	163	181	6·8
12.30–1.0 . . .	30	32·68	20	40	116	23·2	46·4	314	62·8	125·6	172	5
1.0–1.30 . . .	30	39·1	45	90	194	87·1	174·2	305	137·3	274·6	448·8	4·5
1.30–2.0 . . .	30	..	11	22	198	21·8	43·6	437	48	96	139·6	4
CASE 2.												
7.40–8.10 . . .	30	..	20	40	376	75·2	150·4	450	900	180	330	5
8.10–8.40 . . .	30	..	44	88	160	70·5	141	265	117	234	375	6
8.40–9.20 . . .	40	..	24	36	450	108	150	729	175	283	433	5
9.20–10.10 . . .	50	..	26	31·2	477	124	149	963	250	300	449	5
Meal 10.10–10.30												
10.10–10.50 . . .	40	..	12	18	349	41·8	63	861	103	153	216	5
10.50–11.40 . . .	50	..	12	14·4	343	41·2	49·4	108·9	131	157	206·4	5
11.40–11.55 . . .	15	..	15	60	375	56·2	224·8	108·5	164	656	880·8	5
11.55–1.30 . . .	95	..	56	35·5	483	270	171	768	430	272	443	5
1.30–3.0 . . .	90	..	47	31·5	608	285	190	702	330	220	410	4·5
3.0–3.40 . . .	40	..	18	27	626	113	170	833	149	223	393	4·5
3.40–4.10 . . .	30	..	13·5	27	640	86·5	177	903	122	244	421	4
4.10–4.40 . . .	30	..	14	28	650	91	182	953	134	268	450	4
CASE 3.												
11.30–7.30 . . .	480	..	160	20	800	283	160	112	1792	224	384	5
P.M.—Meal 7.30–7.45												
7.30–10.5 . . .	155	..	65	25·2	160	104	40·3	520	338	131	171·3	5·5
10.5–10.35 . . .	30	..	10	20	140	14	28	640	64	128	156	6
10.35–11.5 . . .	30	..	10	20	140	14	28	280	28	56	84	6·5
11.5–11.35 . . .	30	..	10	20	−8·0	−0·8	−1·6	30	3	6	4·4	6·8
11.35–12.0 . . .	25	..	10	24	150	15	36	63	63	152	188	6
Meal 12.0–12.25												
12.0–12.30 . . .	30	..	8	16	320	25·5	51	700	56	112	163	6
12.30–1.0 . . .	30	..	14	28	560	78	156	520	73	146	302	5·5
1.0–1.30 . . .	30	..	7	14	1520	106	212	400	28	56	268	5
1.30–2.0 . . .	30	..	11	22	720	79	158	860	94·5	189	347	5
2.0–2.30 . . .	30	..	12	24	800	96	192	880	156	312	504	5
2.30–3.0 . . .	30	..	11	22	1200	132	264	440	48·5	97	301	5
3.0–3.30 . . .	30	..	11	22	400	44	88	740	81·5	163	251	4·5
3.30–4.0 . . .	30	..	12	24	450	54	108	1100	122	244	352	5
4.0–4.30 . . .	30	..	9	18	480	44·2	88·4	920	82·5	165	253·4	5

TABLE I—*continued.*

	Minutes.	Alv CO_2 mm.Hg.	Volume.		Acidity (Ph 7·4) c.c. N/100 alkali.			NH_3 c.c. N/100.			A+N per hour.	pH.
			c.c. total.	c.c. per hour.	Per cent.	In whole amount.	Per hour.	Per cent.	In whole amount.	Per hour.		
CASE 4.												
9.45–6.45	600	..	175	17	515	901	90	640	1120	112	202	5
6.45–7.15	30	..	17	34	365	62	124	522	89	178	302	4·5
Meal 7.15–7.30												
7.15–7.45	30	..	12	24	262	31·9	63·8	462	55·3	110·6	174·4	5
7.45–9.30	105	..	106	61	91	96·4	55	237	251	144	199	5·5
9.30–10.0	30	..	37	74	−18	−6·3	−12·6	75	27·7	55·4	42·8	7
10.0–10.30	30	..	19	38	100	19	38	95	18	36	74	6·8
10.30–11.0	30	..	20	40	163	32·6	65·2	130	26	52	117·2	6
11.0–11.30	30	..	20	40	272	54·2	108·4	244	48·8	97·6	206	55
11.30–12.0	30	..	22	44	398	87·8	175·6	339	74·5	149	324·6	5
12.0–12.30	30	..	24	48	340	81·5	163	444	106·8	213·6	376·6	4·5
Meal 12.30–1.0												
12.30–1.0	30	..	22	44	322	70·7	141·4	324	71·2	142·4	283·8	4·5
1.0–1.30	30	..	29	58	431	125	250	564	163·5	327	577	4·5
1.30–2.0	30	..	7	14	398	27·9	55·8	237	16·6	33·2	89	4·5
2.0–2.30	30	..	11	22	547	60	120	620	68·1	136·2	256·2	4·5
2.30–3.0	30	..	13	26	585	76	152	644	83·5	167	319	4·5
3.0–3.30	30	..	7	14	870	61	122	300	21	42	164	4·5
3.30–4.0	30	..	19	38	681	129·5	259	283	53·5	107	366	4·5
Meal 4.0–4.30												
4.0–4.30	30	..	20	40	597	119·4	238·8	104·2	20·8	41·6	280·4	4·5
CASE 5.												
8.20–11.45	205	42·29	145	42·4	123	178	52·1	315	457	133	185·1	..
Meal 12.15–12.30												
11.45–1.0	75	45·58	52	416	−210	109	87	98	51	41	128	..
1.0–1.30	30	43·05	42	84	232	97	194	201	84	168	362	..
1.30–2.0	30	40·01	23	46	352	81	162	319	73	146	308	..
2.0–2.30	30	36·96	14	28	540	76	142	517	72·4	145	287	..
2.30–3.0	30	43·43	15	30	644	966	193	863	129	258	451	..
3.15	..	42·29	..	..	..	..	..	..	..	..	..	..
CASE 6.—Starvation Control.												
10.5–8.5	600	..	200	20	127	254	25·4	191	382	38·2	63·6	..
8.5–9.5	60	..	60	60	380	228	228	890	534	534	762	..
9.5–10.5	60	..	130	130	500	650	650	460	598	598	1248	..
10.5–11.5	60	..	80	80	500	400	400	430	344	344	744	..
11.5–1.5	120	..	100	50	233	233	116·5	330	330	165	281	..
Case 6.—Second Exp., Meal taken between first and second specimens.												
6.45–9.45	180	..	180	60	544	979	326·4	1044	1879	626·4	952·8	..
Meal 9.45–10.0												
9.45–11.15	90	..	120	80	104	124·8	82·2	344	412·8	275·2	358·4	..
11.15–12.15	60	..	180	180	52	93·6	93·6	144	259·2	259·2	342·8	..
12.15–1.15	60	..	55	55	37	20·35	20·35	20·4	11·2	1·2	31·5	..

In all these experiments there was either a very slight decrease or none at all in the A + N value. According to the results obtained from the all-day experiments, this would be ascribed to the latter part or acid tide of the breakfast effect.

(2) *The lunch or mid-day meal effect.*—To be sure that this meal alone is influencing the urine the subject must be starved until lunch time. Owing to the difficulty in inducing the breakfastless subject to starve until the usual lunch hour this meal was taken a little earlier than usual. Case 2 in the table is one of a series of three such experiments. The experiment commenced at 7.40 a.m.

Half-hourly specimens were collected, and the meal was taken between 12 and 1 o'clock. It will be seen that during the period of starvation the acid and NH_3 per hour steadily increase, but following the meal they begin to decrease after the first specimen, thus showing an alkaline tide. A starvation experiment was performed, the results being seen in the table, Case 6. The subject starved from 10.5 p.m. to 1.5 p.m. on the next day, hourly samples being collected after 8.5 a.m. The acidity and NH_3 per hour increase steadily for the first three hours, and then decrease, in striking contrast to the next experiment, performed on the same subject on a different day. The times were roughly the same; only a meal was taken between the first and second samples. The acidity and ammonia per hour at once fall after the meal, and continue to do so to the end of the experiment. The pH of the urine shows a remarkable rise after a meal, followed by a fall. As has been noted by many observers, it bears little or no relation to the titrable acidity of the urine.

From what has been described it can be seen that the cases examined show the alkaline tide, as first described by Bence Jones. This tide is only apparent or constant as a result of the first meal of the day. Following the alkaline tide is an acid tide, which continues from three to four hours, thus occurring in the usual lunch time. If a meal is taken during this period, the alkaline tide which would normally follow it is overshadowed, and shows itself usually by a very slight decrease in acidity. The proof that these changes are associated with meals depends on the fact that they are abolished by starvation. The alkaline tide finds a ready explanation in the secretion of gastric juice, and the acid tide in the secretion of alkaline pancreatic juice, yet there is no absolute proof of this.

Observations from a gastrectomised man were so irregular as to be worthless, and are not reproduced.

The sequence of changes fits in very well with the traditional explanation, and, since a diurnal origin is excluded by the starvation experiments, this is probably the true explanation. It was hoped that by examining men who secreted varying quantities of HCl, quantitative urinary changes would be found. Unfortunately this was not found to be the case. When the multitude of factors acting upon the urinary constituents is considered, it is not to be wondered at that the curves are rough and the changes uneven. The general metabolic processes tend in themselves to vary the reaction. Again, there is the secretion of a large quantity of gastric juice, which will tend to concentrate the urine and thus throw out calculations. That the volume of urine decreases immediately after a meal is easily seen from the tables. The juice is absorbed again in the intestines together with imbibed fluid, and again the calculations are upset. Yet through all this a definite alkaline tide appears to follow the first meal of the day.

SUMMARY.

(1) By examining specimens of urine before and after meals, it was found that the first meal of the day was followed by a decrease in the excretion of acid and ammonia calculated per hour. This alkaline tide lasted one to two hours, and was followed by a period of increased excretion of acid and alkali per hour. This acid tide lasted for about two hours, the period of the whole meal effect taking roughly from four to five hours.

(2) The changes following lunch were obscured, in the majority of cases, by the acid tide of the breakfast.

(3) Diurnal origin of these variations was excluded by starvation experiments.

(4) In view of these results, the alkaline tide was associated with gastric secretion and the acid tide with pancreatic secretion.

(5) The pH of the urine was found to rise after a meal, and to fall later.

This investigation was commenced in collaboration with Dr. E. L. Kennaway, who performed some of the urinary analyses, which I publish with his permission. I have to thank him for his advice and help throughout the investigation.

REFERENCES.

Bence-Jones.—(1898) Quoted in Schaefer's 'Text-book of Physiology,' p. 579.
Bennett, T. I., and Ryle, J. A.—(1921) *Guy's Hosp. Rep.*, **71**, 268.
Campbell, J. A.—(1920) *Biochem. J.*, **45**, 5.
Folin, O.—(1919) 'Manual of Biological Chemistry,' p. 97.
Leathes, J. B.—(1919) *Brit. Med. J.*, **2**, 165.
Malfatti, H.—(1908) *Zeitschr. f. anal. Chem.*, **47**, 273.
Quincke.—(1884) *Zeitschr. f. klin. Med.*, **7**, Suppl. 22.

TESTING THE ANTIGENIC VALUE OF DIPHTHERIA TOXIN-ANTITOXIN MIXTURES.

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Toxin-antitoxin mixtures are now being widely used for the production of immunity against diphtheria. Park (1922) in New York alone has immunised 34,000 children, and in England also the practice has been adopted, O'Brien, Eagleton and Okell (1923) having already immunised some hundreds. In view of this extensive use it is obviously important that something should be known of the antigenic value of the mixtures employed.

Although Park and Zingher and others have studied the immunising efficiency of different mixtures upon groups of children, no practical laboratory method of standardisation has been available, and so far these mixtures have been standardised only according to their toxicity. The original requirements of the U.S.A. authorities were that a mixture should contain 3 L+ doses of toxin per c.c., so neutralised that 1 c.c. injected subcutaneously was harmless to guinea-pigs, while guinea-pigs injected with 5 c.c. should survive at least 10 days, but ultimately die of paralysis. These three requirements may be taken as (*a*) a standard of concentration, (*b*) a test to ensure that the mixture is not toxic in reasonable doses, and (*c*) that the toxin has not been so over neutralised as to destroy all antigenic value. It may be stated here that more toxic measures are now used in America. Park (1922) states: "Dr. Banzhaf now standardises our preparation so that 1 c.c. causes paralysis in the majority of guinea-pigs weighing 250 grm.; in all 5 c.c. causes paralysis which ends in death."

It is probable that, owing to the variations in the constitution of different toxins, the antigenic value of a mixture cannot, with any certainty, be judged by its toxicity. Efforts have therefore been made to standardise the actual antigenic value of mixtures by the rate and extent of antibody production following their injection. Four such methods of standardisation are described below.

METHOD A.

The most obvious method of testing the antigenic value of a toxin-antitoxin mixture would be to inject the mixture into experimental animals, and then to test how soon such animals were able to tolerate living culture or toxin. Tests for tolerance have the disadvantage that many test animals are needed to give quantitative results. A convenient modification of this method is to inject rabbits with the mixture under test, to withdraw blood at stated intervals, and to titrate the serum against toxin; the immunity produced by the injections of the toxin-antitoxin mixture is judged by the antitoxin produced rather than by the tolerance gained. Table I records the "primary stimulus response" of several rabbits to a single injection of four different toxin-antitoxin mixtures. Each normal rabbit was injected subcutaneously with 5·0 c.c. of the mixture under test; a few c.c. of blood were withdrawn each week or twice a week, and the antitoxic value of the blood titrated by the intracutaneous method. Rabbit G 21 eventually produced over one-half of a unit of antitoxin per c.c. of serum as the result of the subcutaneous injection of a particular mixture; two weeks elapsed before any antitoxin was detectable, and the antitoxic value gradually increased over a period of eight weeks. That the particular mixture used in this test was a good antigen was capable of easy and moderately rapid proof. The mixtures tested on the other rabbits did not produce such rapid and such marked results. Rabbits G 18 and G 23 had produced only small traces of antitoxin, difficult to detect, by the fourth week after injection, and the highest value reached was approximately $\frac{1}{30}$ and $\frac{1}{100}$ unit respectively. The fourth mixture tested failed to produce in G 20 any detectable antitoxin; this alone is not sufficient to condemn the mixture as having no antigenic value, because the existence has already been demonstrated of active immunity to diphtheria in the absence of detectable antitoxin (Glenny and Allen, 1922[1]). Rabbit G 20 continued to show no antitoxin until at the end of 14 weeks it was injected intracutaneously with a Schick dose of diphtheria toxin. Four days later the serum of this rabbit contained $\frac{1}{50}$ unit of antitoxin per c.c.; by the ninth day the antitoxic value had reached 0·18 unit. The use of the Schick test as a "secondary stimulus" (Glenny and Allen, 1922)[2] renders active immunity more easy to detect. Rabbit G 46 in Table I affords an illustration of this method of magnifying the result of the primary stimulus. Four weeks after the injection of a toxin-antitoxin mixture the serum of rabbit G 46 contained $\frac{1}{500}$ unit per c.c.; many careful titrations are needed to confirm the existence of such small amounts of antitoxin. A Schick dose of toxin was injected, and within a week the antitoxic content had reached 0·15 unit.

TABLE I.—*Showing the Antitoxic Content of the Blood of Five Rabbits after the Injection of a Toxin-Antitoxin Mixture as a Primary Stimulus.*

Rabbit	G 21	G 18	G 23	G 20	G 46
No. of mixture	1	2	3	4	5
	Units of antitoxin per c.c.				
Before injection	*nil*	*nil*	*nil*	*nil*	*nil*
1 week after	,,	,,	,,	,,	,,
1½ weeks after	—	—	—	,,	—
2 ,, ,,	0·02	*nil*	*nil*	,,	*nil*
2½ ,, ,,	0·18	—	—	,,	—
3 ,, ,,	0·22	*nil*	0·001	,,	0·001
3½ ,, ,,	0·22	—	—	—	—
4 ,, ,,	0·22	0·004	0·0015	*nil*	0·002[A]
5 ,, ,,	0·25	0·02	0·0015	,,	0·15
6 ,, ,,	0·45	0·03	0·011	,,	0·07
7 ,, ,,	0·45	0·03	0·011	,,	0·045
8 ,, ,,	0·55	0·035	—	,,	—

Nil indicates that no antitoxin could be detected, *i. e.* less than 0·0005 unit per c.c. was present.

[A] = injection of a Schick dose of toxin.

METHOD B.

Table II gives further illustrations of this method of magnifying the primary stimulus response. Seven rabbits were each injected subcutaneously with 1 c.c. of the same toxin-antitoxin mixture. At the end of four, eight and twelve weeks pairs were reinjected, one with a Schick dose of toxin and the other with a second dose of 1 c.c. of the same toxin-antitoxin mixture (T.A.M.). In each rabbit a marked increase in antitoxic value occurred within a week of the second injection, indicating that each rabbit was actively immune as the result of the antigenic action of the toxin-antitoxin mixture. It is clear from Table II that the primary stimulus response to the same dose of the same mixture may vary in different rabbits. At the end of eight weeks G 35 and G 39 both showed traces of antitoxin, while G 34, G 36 and G 38 showed none; at the end of twelve weeks rabbit G 36 showed no antitoxin, G 38 showed $\frac{1}{500}$ unit, and G 39 showed $\frac{1}{60}$ unit as a result of the primary stimulus alone. The table also shows that in each of the three pairs of rabbits tested a single Schick dose of toxin gave a better secondary stimulus response than 1 c.c. of a toxin-antitoxin mixture. The table also shows how it is simple to demonstrate in the same animal the antigenic properties of a mixture, both as a primary and as a secondary stimulus. It is of interest to record that G 34 responded to an injection of a Schick dose of toxin by the production of at least 100,000 times as much antitoxin as would be necessary completely to neutralise the toxin injected.

TABLE II.—*Showing the Antitoxic Content of the Blood of Seven Rabbits after the injection of a Primary Stimulus consisting of a Toxin-Antitoxin Mixture, and a Secondary Stimulus (one, two and three months later), consisting of a Schick dose of Diphtheria Toxin or of another Toxin-Antitoxin Mixture.*

Rabbit	G 32	G 33	G 34	G 35	G 36	G 38	G 39
Primary stimulus	1·0 B. 346	1·0 B. 346	1·0 B. 346	1·0 B. 346	1·0 B. 346	1·0 B. 346	1·0 B. 346
Number of weeks elapsing between primary and secondary stimulus	4 weeks	4 weeks	8 weeks	8 weeks	12 weeks	12 weeks	—
Secondary stimulus	Schick	T.A.M.	Schick	T.A.M.	Schick	T.A.M.	—
	Units of antitoxin per c.c.						
3 weeks after primary stimulus	*nil*	*nil*	*nil*	*nil*	*nil*	*nil*	*nil*
4 weeks after	"	"	"	"	"	"	"
	injected	injected					
5 " "	0·08	0·1	"	"	"	"	"
6 " "	0·12	0·05	"	"	"	"	"
7 " "	—	—	"	0·002	"	"	0·002
8 " "	0·05	0·01	"	0·006	"	"	0·002
			injected	injected			
9 " "	—	—	1·0	0·12	"	"	0·005
10 " "	—	—	1·75	0·12	"	"	0·005
11 " "	—	—	—	—	—	0·001	0·005
12 " "	—	—	—	0·04	*nil*	0·002	0·016
					injected	injected	
13 " "	—	—	—	—	0·06	0·05	0·02
14 " "	—	—	—	—	·1	0·03	0·02

Nil indicates that no antitoxin could be detected, *i. e.* less than 0·0005 unit per c.c. was present.

METHOD C.

One disadvantage of testing antigenic values by means of the primary stimulus response is the length of time elapsing before such a response occurs.

This disadvantage is lessened by using the modification recorded above in Table II, but more time can be gained by testing mixtures for the secondary stimulus response only in rabbits already immune. An increase in antitoxin content is demonstrable in four days, and high unit values are reached in from six to ten days. The advantages of this method are the rapidity with which a result can be obtained, and the great increase in the ease and accuracy of testing gained through the production of higher unit values. A marked disadvantage of the method is the introduction of additional variable factors.

TABLE III.—*Showing the Antitoxic Values of Three different Immune Rabbits after Secondary Stimuli consisting of a number of injections of the same Toxin-Antitoxin Mixture.*

Rabbit . .	G 8				G 11			G 17		
Number of test .	1	2	3	4	1	2	3	1	2	3
Weeks since last injection of same antigen	—	15	6	8	—	6	8	—	6	8
Before injection .	0·01	0·0045	0·025	0·01	0·009	0·16	0·14	0·07	0·16	0·12
1 day after .	—	—	0·018	—	—	0·14	—	—	0·14	—
2 days after .	—	0·0045	0·018	0·008	0·006	0·14	0·14	0·06	0·12	0·11
3 ,, ,, .	—	—	0·04	—	—	0·14	—	—	0·12	—
4 ,, ,, .	0·5	0·08	0·14	0·18	0·22	0·3	0·45	0·4	0·22	0·33
5 ,, ,, .	—	—	—	—	—	—	—	—	—	—
6 ,, ,, .	—	0·55	0·7	0·55	1·0	0·7	0·7	1·0	0·6	0·55
7 ,, ,, .	2·0	—	—	—	—	—	—	—	—	—
8 ,, ,, .	—	0·4	0·5	0·35	0·9	0·7	0·8	0·9	0·6	0·55
9 ,, ,, .	—	—	—	—	—	—	—	—	—	—
10 ,, ,, .	—	0·25	0·35	—	0·7	0·7	0·7	0·6	0·55	0·55
11 ,, ,, .	1·5	—	—	—	—	—	—	—	—	—
12 ,, ,, .	—	—	—	—	—	—	—	—	—	—
13 ,, ,, .	—	0·22	0·3	0·22	0·55	0·5	0·45	0·55	0·55	—
14 ,, ,, .	1·0	—	—	—	—	—	—	—	—	—

The extent of a secondary stimulus response depends not only upon the individual capacity of the animal, but also upon the potential activity or capability of increased response induced by former stimuli as well as upon the degree and stage of immunity of the test animal at the time of injection. It is obvious therefore that test animals must be carefully chosen if it is necessary to detect quantitative differences between the antigenic values of two mixtures. Table III records a number of tests upon the secondary stimulus response, induced by the same antigen in three different rabbits. The first test upon rabbit G 8 showed an antitoxic content of two units per c.c.; later tests of the same antigen upon the same rabbit and upon two other rabbits all showed an antitoxic content of between 0·55 and 1·0 units per c.c. The interval of time elapsing between the 1st and 2nd test on G 8 was 15 weeks; between the 2nd and 3rd on G 8 and the 1st and 2nd on G 11 and G 17 was 6 weeks, and that between the 3rd and 4th on G 8 and the 2nd and 3rd on G 11 and G 17 was 8 weeks. The three rabbits recorded in the table exhibit considerable uniformity of response; control of certain variable factors would increase the degree of the uniformity. Thus the last two tests on both G 11 and G 17 were made when the initial values of the rabbits were between 0·12 and 0·16 unit per c.c., and within 6 to 8 weeks of the previous injection; the resultant values were between 0·55 and 0·7 unit on the 6th day, between 0·55 and 0·8 unit on the 8th day, and between 0·55 and 0·7 unit on the 10th day. The readings of the individual rabbits varied even less than this. Rabbit G 8 affords an illustration of individual peculiarity, for the 8th day reading in each case was decidedly less than the 6th day, and the rate of loss after each test was far greater than that in either of the other two rabbits. Five weeks after reaching a value of 0·55 rabbit G 8 had fallen to 0·025 compared with 0·16 unit in both G 11 and G 17 at the same interval of time after a value of 1 unit had been reached in each rabbit. Again, seven weeks after reaching a value of 0·7 unit G 8 had fallen to 0·01 compared with 0·14 and 0·12 in G 11 and G 17 after reaching 0·7 and 0·6 unit respectively. Although Table III affords good evidence that uniformity of response can be achieved, yet great variation may be obtained if conditions are not uniform, for the same dose of the same antigen in another experiment has produced as low a value as 0·12 unit in one rabbit and as high as 6·2 unit in another.

METHOD D.

The methods so far described involve injecting rabbits, bleeding and titrating certain samples of blood against diphtheria toxin, making use of guinea-pigs as indicators. These methods need special laboratory technique and the use of a number of test animals. In the method now to be described the technique is of the simplest and the number of test animals is reduced to a minimum.

For each mixture of toxin and antitoxin prepared for immunisation against diphtheria, it is necessary to determine that a definite dose is non-lethal to guinea-pigs; the size of the dose depends upon the standard of toxicity adopted. According to the earlier American requirements, guinea-pigs should survive 1 c.c. of the mixture. The simplified method of testing the antigenic value of toxin-antitoxin mixtures consists in ascertaining the degree of immunity produced in the surviving guinea-pigs which have been used to demonstrate that the mixture is harmless in certain doses. The simplest method of testing for a certain degree of immunity is to make use of the Schick reaction. In all methods of testing antigenic values it is advisable to limit the primary stimulus to a single injection, consequently the resulting immunity is small, and may not be sufficient to give a negative Schick reaction. If, however, a sufficient degree of active immunity has ensued, each injection of toxin for the Schick test will act as a secondary stimulus (Glenny and Allen, 1922)[2] and raise slightly the degree of immunity, until at some subsequent test the animal shows a negative Schick reaction. Table IV records the results of injecting a number of guinea-pigs with varying doses of a toxin-antitoxin

TABLE IV.—*Showing the Reactions produced upon Guinea-pigs by Schick Doses of Diphtheria Toxin given at varying intervals of time after the Injection of Primary Stimuli consisting of varying amounts of a Toxin-Antitoxin Mixture.*

Guinea-pig	B.	C.	D.	A.	Z.	Y.	S.	O.	N.	R.	P.	W.	X.	K.	H.	F.
Volume of T.A.M. as primary stimulus	0·2	0·2	0·2	0·2	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	5·0	5·0	5·0
	Reaction produced by Injection of Schick Dose of Toxin.															
1 week after primary stimulus	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2 weeks ,, ,,	+	...	...	...	+	...	...	...	...	...	...	...	...	+	...	...
3 ,, ,, ,,	+	+	...	...	+	+	+	...	...	...	...	...	...	+	+	...
4 ,, ,, ,,	+	+	+	...	—	+	±	+	+	...	...	...	...	+	Died	+
5 ,, ,, ,,	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	Died
6 ,, ,, ,,	—	+	+	+	...	—	—	—	—	+	±	...	...	+	...	...
7 ,, ,, ,,	...	+	+	+	...	...	...	...	...	...	±	—	...	+	...	...
8 ,, ,, ,,	...	—	±	±	...	...	...	...	...	?	—	...	+	+	...	...
9 ,, ,, ,,	...	...	—	—	...	...			...	—	...	...	±	+		
10 ,, ,, ,,	...	...	...	...	...	...	...	...	...	...	...	...	—	+	...	...

TABLE V.—*Showing Tests for Toxicity and Antigenic Value of Three Different Toxin-Antitoxin Mixtures.*

Toxin-antitoxin mixture	A.						B.						C.				
Dose in c.c.	1·0	1·0	1·0	5·0	5·0	5·0	1·0	1·0	1·0	5·0	5·0	5·0	1·0	1·0	5·0	5·0	5·0
	Reactions produced by Schick Dose of Toxin.																
1 week after primary stimulus	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2 weeks ,, ,,	...	...	...	Died 11th day	...	...	...	...	...	...	...	...	...	...	...	...	...
3 ,, ,, ,,	+	+	+	...	Died 22nd day	Died 24th day	+	+	+	Died 24th day	Died 24th day	Died 26th day	+	+	+	+	+
4 ,, ,, ,,	—	—	—	...	...	...	+	+	+	...	...	...	+	+	+	+	+
5 ,, ,, ,,	...	...	...	...	...	...	+	+	+	...	...	...	—	+	+	+	+
6 ,, ,, ,,	...	...	...	...	...	...	+	+	+	...	...	...	...	±	+	—	+
7 ,, ,, ,,	...	...	...	...	...	...	—	—	+	...	...	...	...	—	—	...	+
8 ,, ,, ,,	...	...	...	...	...	...	...	...	—	...	...	...	...	...	...	...	+

mixture and then testing them with Schick toxin each week, commencing two, three, four or six weeks after the injection of the mixture. Of four guinea-pigs injected with 0·2 c.c. of T.A.M. all gave at least three positive reactions before their immunity was sufficiently raised to give a negative reaction. Of the nine guinea-pigs injected with 1·0 c.c. of T.A.M. none needed more than two injections of Schick toxin before the reaction became negative. In both groups the degree of immunity represented by a negative Schick reaction was attained at a stage determined by the number of previous injections of Schick toxin, rather than by the time interval after the primary stimulus. We have adopted as a measure of immunity conferred, and consequently of antigenic values of mixtures injected, the number of weekly Schick tests required before a negative reaction occurs, the injections of Schick toxin being commenced three weeks after the primary stimulus. It should be noted that two of the three guinea-pigs injected with 5·0 c.c. of the mixture died of diphtheritic paralysis four or five weeks after injection. The surviving guinea-pig showed signs of paralysis. This guinea-pig failed to respond to injections of Schick toxin and was less immune than those that had received only $\frac{1}{5}$ c.c. of the same mixture. It has been found frequently that excessive doses of T.A.M. producing poor condition fail to produce immunity, and further that in a group of guinea-pigs injected with the same dose of the same antigen, one in poor condition from any cause may respond far less readily than others in the same group.

Table V shows comparative tests for toxicity and antigenic value of three different toxin-antitoxin mixtures. Three guinea-pigs were injected with 1·0 c.c. and three with 5·0 c.c. of each mixture. Mixtures A and B were of similar toxicity; guinea-pigs injected with 5 c.c. of A died in 11, 22 and 24 days, those injected with 5 c.c. of B died in 24, 24 and 26 days. Marked differences were seen in their antigenic values; the three guinea-pigs injected with 1·0 c.c. of A gave negative reactions at their 2nd Schick injection; those that had received 1·0 c.c. of B gave negative reactions at the 5th, 5th and 6th injection. Again, when mixtures B and C are compared, discrepancies are seen between toxicity and antigenic value; C was less toxic than B in that all three guinea-pigs injected with 5·0 c.c. of C survived, yet the two animals receiving 1 c.c. of C showed higher immunity, yielding negative reaction at the 3rd and 5th (reduced at 4th) injection compared with 5th, 5th and 6th with 1 c.c. of B. The guinea-pigs injected with 5 c.c. of C although surviving without signs of paralysis were sufficiently affected in general condition to fail to respond more readily than those injected with 1 c.c.

SUMMARY.

Evidence is brought forward to show that the toxicity test alone may give incomplete or erroneous conclusions as to the immunising value of a toxin-antitoxin mixture intended for immunisation against diphtheria.

Four methods are described for testing the antigenic value of mixtures:

(*a*) Tracing the antitoxic value of the blood of rabbits after a single subcutaneous injection of the mixture as a "primary stimulus."

(*b*) Accelerating the results of (*a*) by a subsequent injection, as a

"secondary stimulus," of either the same mixture or of a Schick dose of toxin.

(*c*) Tracing the increase in antitoxic values of the blood of immune rabbits after a subcutaneous injection of the mixture as a secondary stimulus.

(*d*) Injecting guinea-pigs, which have survived the test for toxicity of the mixture, with Schick doses of diphtheria toxin at weekly intervals commencing three weeks after the injection of the mixtures.

REFERENCES.

Glenny, A. T., and Allen, K.—(1922)[1] *J. Hyg.*, **21**, 100.—(1922)[2] *Ibid.*, **21**, 104.

O'Brien, R. A., Eagleton, A. J., Okell, C. C., and Baxter, M.—(1923) *Brit. J. Exper. Pathol.*, **4**, 29.

Park, W. H.—(1922) *J. Amer. Med. Assoc.*, **79**, 1584.

THE "GASTRIN" CONTENT OF THE HUMAN PYLORIC MUCOUS MEMBRANE.

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Assuming that the human pyloric mucosa yields a gastric excitant or gastrin on acid extraction, similarly to other mammals, we intended to examine the gastrin content of the mucous membrane in pathological conditions and compare it with the normal. We use the term "gastrin," not in the sense in which it was coined by Edkins (1905, 1906), viz. to denote a gastric hormone, but merely as an excitant obtainable from the stomach and capable of stimulating gastric secretion on intra-vascular injection.

It was unfortunate, but unavoidable, that the material could only be obtained from the post-mortem room many hours after death.

The pyloric mucous membrane was dissected off, and on most occasions prepared by boiling with HCl, etc., as for the extraction of secretin. In a few cases the method of Dale and Laidlaw (1912) was utilised.

We employed the method devised by one of us (1922)[1] for the observations on the gastric secretion, the experimental animal being the cat.

The results are shown in Table I.

TABLE I.

	Condition of stomach.	Condition of individual.			No. of hours between death and preparation of extract.	Method of extraction.	Effect on gastric secretion.
		Age.	Sex.	Disease.			
1	Pylorus normal	?	F.	Carcinoma (cervix uteri)	48 hours	HCl	No secretion.
2	Pylorus normal: recent ulcers in fundus	70	M.	Lymphosarcoma (mediastinum)	48 „	„	Marked secretion.
3	Pylorus normal	62	F.	Carcinoma (pelvic colon)	14 „	„	No secretion.
4	Pylorus congested	60	F.	Endocarditis	59 „	„	„
5	Pylorus normal	35	M.	$CHCl_3$; cardiac failure	50 „	„	Slight secretion.
6	„ „	41	F.	Mastoid abscess pyæmia	12 „	„	No secretion.
7	„ „	56	M.	Chronic interstitial nephritis	28 „	D. & L.	„
8	„ „	34	M.	Meningitis	24 „	D. & L.	Secretion ?
9	„ „	18	F.	Acute lupus erythematosus	63 „		
10	„ „	64	M.	Myocarditis and nephritis	36 „		

HCl, extraction by boiling with 0·4 per cent. HCl, etc.; D. & L., extraction by the method of Dale and Laidlaw (1912).

From Table I it will be seen that with one exception (Case 2, Fig. 1) all our extracts showed little or no activity. They were not nearly so active as similar preparations from smaller animals, viz. the cat and dog. The exception mentioned was prepared from a stomach which showed signs of recent acute ulcers. This was the only stomach showing ante-mortem local changes.

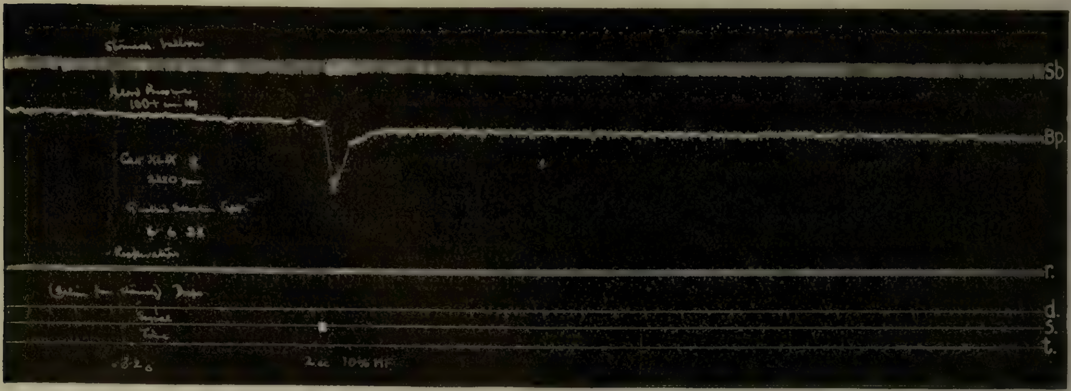

FIG. 1.—Tracing showing the effect of injecting 2 c.c. of pyloric extract from Case 2. The injection was given just after the stomach had been washed out with saline. The drops indicated on the left of the tracing are residual drops of saline. *sb*, balloon tracing; *bp*, blood-pressure; *r*, respiration; *d*, drops of secretion from stomach; *s*, signal; *t*, time in minutes.

It would appear from the negative character of our results either that there is little gastric excitant normally present in the human pyloric mucosa, or that the excitant becomes destroyed by the changes occurring after death. Against the latter argument is the commonly accepted opinion that the mortifying intestine engenders the formation of histamine, a base which should have given us some effect on the gastric secretion (Lim, 1922²). Apparently no histamine was formed. At any rate, post-mortem changes had not affected the activity of the preparation made from Case 2 forty-eight hours after death. It may be that the presence of the gastric excitant is in some way related to the ulceration found in the stomach. It would seem, therefore, that the human pyloric mucosa contains little or no gastrin, and certainly much less gastrin than is found in the lower animals.

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REFERENCES.

Dale, H. H., and Laidlaw, P. P.—(1912) *J. Physiol.*, **44**, xi.
Edkins, J. S.—(1905) *Proc. R. Soc.*, B, **76**, 376.—(1906) *J. Physiol.*, **34**, 133.
Lim, R. K. S.—(1922)¹ *Quart. J. Exp. Physiol.*, **13**, 71.—(1922)² *ibid.*, **13**, 79.

THE SCHICK TEST AND ACTIVE IMMUNISATION.

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It will probably be some considerable time before the people of any large city in England are so alive to the importance of the more recent measures for the control of diphtheria that they will freely allow their children to be tested by the Schick method, and if found to be positive reactors to be given the three doses of a toxin-antitoxin mixture. In the remarkable campaign carried out recently in New York some ninety thousand children were tested and if positive immunised, 70 per cent. of the parents giving consent.

However, these new measures are slowly but surely being adopted in England, and a review of the results obtained is probably sufficiently important to merit publication at this stage. Leete (1920), Ward (1921), Dickinson (1922), Harries (1922), Dudley (1922), and Copeman with the present authors (1922) have given the results of Schick tests.

To these series we have to add tests on 2769 individuals (see Table I).

TABLE I.—*Results of Schick Tests.*

Adults.	Number tested.	Percentage negative.
Medical students—a London teaching hospital	160	35
Nurses—a London teaching hospital . .	48	49·5
Nurses—School A	182	78
„ „ B	59	74
Children.		
School B	739	72
„ C	329	69
An institution for children	1046	62
Good class private school	53	28
Infant welfare centre	153	24

The results closely resemble those obtained elsewhere, the groups from a "higher" social stratum showing a lower degree of immunity than those from a "lower," while the children, mostly aged from one to five years, from the infant welfare centre came from a district in which there has been but little diphtheria for some time past.

Apparently the children of the well-to-do are carefully guarded in their early lives, and escape the chance of the frequent mild attacks of unrecognised diphtheria which would otherwise eventually cause them to become immune. In a similar manner the children of intelligent artisans who live in well-kept homes in a district where few cases of diphtheria have occurred in recent years "escape" or "avoid" natural immunisation.

Age distribution.—Our general results agree with those reported in the literature, the immunity rate rising during the years of childhood and becoming constant in early adult life. The deviations in each individual group are associated with the special conditions of the group, or in some cases are probably due to the smallness of the statistical units.

Familial immunity.—As observations accumulate it may become possible to decide whether there is a family factor in the readiness with which immunity is developed. Unfortunately the observations hitherto recorded are insufficient to throw much light upon this point. When immunising 107 families, we found in 20 that the children were positive reactors; in 54 all negative; in 20 the older negative and the younger positive in accordance with Zingher's observations; and in 13 (12 per cent.) the older children positive, while the younger were negative. The members of these 13 families did not live together during the whole of their lives.

In one group of 136 children, all of whom originally gave a Schick-positive reaction, there were only three individuals who failed to give a negative Schick reaction within 11 weeks of the commencement of the "immunisation"; of these two were brother and sister, and both proved refractory until 21 weeks had elapsed. The remaining one was still Schick-positive after this interval.

Twins.—We tested three pairs of twins; each twin gave the same reaction as the other in two cases; the twins were similar and less than three years old. Of a third pair of dissimilar twins, aged 7 years, the boy was a positive reactor and the girl negative.

RESULTS OF IMMUNISATION.

The routine procedure was to give a weekly intramuscular injection of 1 c.c. of toxin-antitoxin mixture until three doses had been given. At different intervals after the last injection small groups of these children, as they became available, were subjected to the Schick test.

TABLE II.—*Results of Immunisation.*

	Weeks elapsed between last injection of toxin-antitoxin mixture and first negative response to Schick test.							
	1	2	3	4	5	6	7	8
School B:								
Initial immunity rate 72 per cent.								
Number tested	19	56	37	4	20	3	1	1
,, negative	17	46	31	4	16	3	1	1
Percentage negative	89	82	84	100	80	100	100	100
School C:								
Initial immunity rate 69 per cent.								
Number tested	—	—	—	13	38	30	15	5
,, negative	—	—	—	9	32	24	15	5
Percentage negative	—	—	—	70	84	80	100	100
Institution for children:								
Initial immunity rate 62 per cent.								
Number tested	12	24	30	53	9	133	2	—
,, negative	6	14	24	29	8	87	2	—
Percentage negative	50	58	80	54	89	65	100	—

The groups are very small and the statistical error therefore great.

A summary of these results is given in Table III.

TABLE III.

Place.	Initial immunity percentage.	No. of positive reactors immunised and re-tested.	No. giving negative Schick test.	Weeks since last injection of mixture.	Percentage Schick-negative.
Institution for children	62	348	296	11	83
School B	72	136	133	11	98
School C	69	101	92	8	91
	—	—	101	20	100

It may be of value to present the evidence in another way.

A group of 136 "positive reactors" was injected and re-tested. Within

8 weeks 129, *i. e.* 94 per cent., showed a negative Schick reaction; thereafter the results were as follows, the figures in brackets giving the number yielding a Schick-negative reaction at the expiry of the corresponding number of weeks: 9 (2), 10 (1), 11 (1), 21 (2). One child, though reinjected, remained positive; we hope to be able to re-examine him later.

With regard to the permanence of the negative reaction in immunised children, we had the opportunity of re-testing a group of 203 children, both those originally negative to the Schick test, and also those who had become negative after immunisation. When re-tested 11 weeks later the reactions agreed in 201 cases; 2 who had been negative now gave a positive reaction. Inquiry revealed that these children had had antitoxin injected some few weeks before the first test was carried out.

To one question of interest we have only a partial answer. From our general knowledge of immunity it is to be expected that a school population showing a high percentage of Schick-negative reactors and having a recent history of diphtheria would be one easily immunised. This anticipation is realised in the large groups of figures given by Zingher. We know also from Dudley's careful work (1922) that a population of boys exposed to an epidemic of diphtheria will show a high percentage of "Schick-negative reactors." It would be of great scientific interest to know how many of the children would have become immune without the injection. Probably some few of them would. We have had unfortunately, in this, as in so many other aspects of this work, very restricted opportunities of making observations under satisfactory conditions; however, at one school, 19 children who gave Schick-positive reactions did not become available to us again for seven months, during which time the remainder of the positive reactors in the school were immunised. All of these 19 children were now injected. Five weeks after the completion of the course of injection these children were again tested; 5 of them gave a positive response. Thus, this group of 19 children who had been in contact with the other children and had also recently had prophylactic injections showed an immunity rate of only 74 per cent., whereas the children who had been fully immunised at this time gave a 99 per cent. immunity rate. Though the figures are small, they indicate that the increased immunity rate in the whole school was almost entirely the result of the toxin-antitoxin injections.

The figures from the welfare centre may be of further help. Of these children only 24 per cent. were Schick-negative reactors when they were first examined. Of 33 children tested, found positive and injected with mixtures, 31, *i.e.* 93 per cent. became Schick-negative reactors within 12 weeks. During this period no cases of diphtheria were notified within the area. Small groups of fresh children were still being tested each week, and the immunity rate was still between 25 and 30 per cent.

It is reasonable to conclude that in this particular population, the greater part of the conversion of the "Schick-positive" population to a "Schick-negative" one was due to the injections given.

Reactions after injection of toxin-antitoxin mixture.—The reactions in these groups of children have been very slight: with the exception of three instances in our early work we have no record that any child developed a reaction and illness sufficiently severe to cause it to be confined to bed for one day. The great majority suffered nothing but a temporary slight soreness of the arm, and very few had any general reaction.

SUMMARY.

(1) The results of somewhat over 2700 Schick tests are given. The percentage immunity at various ages agrees generally with observations in the literature. The " higher " the social status of an urban population group, the lower will its immunity rate be.

(2) The results of the immunisation of 585 positive reactors are given. Between 85 per cent. and 98 per cent. of the positive reactors in the different groups became negative to the Schick test in less than three months.

(3) No serious reactions after injections of the toxin-antitoxin mixtures were observed amongst these children.

REFERENCES.

Copeman, S. M., O'Brien, R. A., Eagleton, A. J., and Glenny, A. T.—(1922) "Experiences with the Schick Test and Active Immunisation against Diphtheria," *Brit. J. Exper. Pathol.*, **3**, 42.

Dickinson, T. E.—(1922) "The Schick Test for the Determination of Susceptibility to Diphtheria: a Record of 1200 Cases," *Lancet*, **1**, 312.

Dudley, S. F.—(1922) "The Relation of Natural Diphtheria Antitoxin in the Blood of Man to previous Infection with Diphtheria Bacilli," *Brit. J. Exper. Pathol.*, **3**, 204.

Harries, E. H.—(1922) "The Carrier of Diphtheria Bacilli from the Fever Hospital Standpoint," *Med. Officer*, **27**, 91.

Leete, H. M.—(1920) "The Schick Reaction," *Lancet*, **1**, 192.

Park, W. H.—(1922) "Toxin-antitoxin Immunisation against Diphtheria," *J. Amer. Med. Assoc.*, **79**, 1584.

Ward, G.—(1921) "The Schick Reaction," *Brit. Med. J.*, **1**, 928.

THE DIAGNOSTIC VALUE OF RABBIT INOCULATION IN *ENCEPHALITIS LETHARGICA.*

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MANY cases of *encephalitis lethargica* present considerable difficulty in their diagnosis, and it was with the idea of overcoming this that the following observations were made. The amount of material at my disposal was small, but it is thought desirable that the investigations should be put on record.

The work may be regarded from two standpoints:

(1) The relative infectivity of the brain emulsions as compared with the cerebro-spinal fluid.

(2) The significance to be attached to the histological changes found in the rabbit brain.

In a former article (1918) I pointed out the general insusceptibility of the lower monkeys to the virus of *encephalitis lethargica*—a condition of affairs which is just the opposite of what occurs in poliomyelitis. Some two years ago Loewe, Hirshfeld and Strauss (1919) showed that *encephalitis lethargica* could readily be transmitted to rabbits by intracerebral inoculation, and my own observations (1920) have convinced me that the rabbit is a much more suitable animal than the monkey, hence all the experiments detailed below were performed on rabbits.

For the injections young rabbits of about 1500 grm. were used, and the inoculations were made intra-cerebrally in the following manner: A small incision was made to the left of the middle line and just behind the level of the mid-interocular line. A small hole was then made by means of a drill (1·0 mm.) and 0·25 to 0·5 c.c. of the suspected brain emulsion injected. The emulsion was prepared by emulsifying about 5 grm. of brain tissue in 20 c.c. of normal saline. Pieces of the basal nuclei removed *post mortem* were preserved in 33⅓ per cent. glycerine till required. In the case of cerebro-spinal fluid 0·5 c.c. was injected intra-cerebrally and 4 c.c. subcutaneously.

The experimental disease in rabbits shows considerable variations in its clinical manifestations; occasionally paralyses, tremors and convulsions are evident, while others die suddenly with no previous sign of ill-health. As a rule the symptoms appear about 48 hours after the injection is made. The temperature is raised 2 to 3° F., the animals are restless and excitable, choreiform movements appear; a day or two later the temperature falls and the animal becomes paretic and dies. The average duration of the infection is five or six days. In a few animals the disease is fulminating and death may occur on the second or third day; in others no symptoms appear at all and the animal is found dead several weeks later.

No lesions are found on macroscopical examination, but microscopically the brain shows changes, the true diagnostic significance of which will be discussed later. The lesions consist of a small round-cell infiltration of the meninges and of the perivascular lymph-sheaths, while small inflammatory foci in the brain substance are not uncommon.

RELATIVE INFECTIVITY OF ENCEPHALITIS MATERIAL.

The results are best given in tabulated form:

Exp. No.	Material.	Identification.	Result of inoculation.	Date.	Remarks.
1	Cerebro-spinal fluid	U. C. H.	Negative	28.12.20	
	Brain emulsion	U. C. H.	Positive	6.1.21	
	Tonsil (filtered)	U. C. H.	Positive	6.1.21	
2	Brain emulsion	M. H.	Negative	6.1.21	Infant. Brain lesion not characteristic. Case recovered.
3	Cerebro-spinal fluid	M. H.	Negative	18.1.21	
4	Cerebro-spinal fluid	M. V.	Positive	4.2.21	One rabbit died 9.9.21, extensive infiltration in brain.
	Brain emulsion	M. V.	Positive	9.3.21	
5	Brain emulsion	T. C.	Negative	29.3.21	Brain infected when received.
6	Brain emulsion	S. M. H.	Positive	29.3.21	

Note.—In every instance the cerebro-spinal fluid was inoculated into Noguchi medium without any evidence of subsequent growth.

From the above table it will be seen that three out of five gave positive results on inoculation when brain emulsions were used, while the cerebro-spinal fluid gave negative results except in one case. One animal, in Experiment No. 4, died seven months after inoculation with the cerebro-spinal fluid, and showed a doubtful infiltration of the meninges. Loewe and Strauss (1920), on the other hand, recorded 12 positive results in 16 cases, but they were working during the height of an epidemic. More recently Kling and collaborators (1921) found changes in the brains of two rabbits which had been inoculated with cerebro-spinal fluid from a case of encephalitis. No clinical symptoms were observed in the animals, but the characteristic lesions were found when the animals were killed 38 and 40 days later. Evidently the animal should be killed some time after inoculation and the brain examined, as if disease results it may be mild and recovery apt to take place.

SIGNIFICANCE OF HISTOLOGICAL LESIONS.

In the earlier part of this article it is indicated that in the diagnosis of experimental encephalitis, owing to the variability of the clinical signs, chief reliance is placed on the histological changes found in the brain. It is proposed here to discuss the true significance of these.

In the first place it has been known for a long time that the rabbit is extremely sensitive to intracerebral inoculations. Thus intracerebral inoculations of small doses of bacteria which are harmless when inoculated elsewhere will produce a rapid and fatal meningitis, while the injection of small amounts of such a relatively harmless substance as sterilised indian ink may produce fatal results in young rabbits.

Quite recently Oliver (1922) has drawn attention to the occurrence of a spontaneous chronic meningo-encephalitis in rabbits. The lesions observed consist of an infiltration of the meninges and of the vascular lymph-sheaths with round cells, chiefly lymphocytes and a few plasma-cells. No micro-organisms were demonstrable in the brain. Oliver draws attention to the similarity of the lesion to that found in man in *encephalitis lethargica.* These infections in rabbits may be related to the spontaneous encephalitis of horses (Borna's disease), which recently has been shown to be transmissible to rabbits by experimental inoculation (Moussu, 1922).

Secondly, other filter-passing viruses tend to produce a somewhat similar histological change, viz. poliomyelitis, rabies, vaccinia, nervous herpes. Poliomyelitis, however, can only be transmitted to rabbits with the greatest difficulty, and when accomplished, many polynuclear leucocytes are to be found in the infiltrations. Rabies is an unlikely possibility, and the previous history of the case from which the material was derived should be sufficient. Vaccinia virus, as was recently shown by Levaditi and Nicolau (1921), can produce changes in the rabbit's brain almost indistinguishable from those of encephalitis, but I have found that although a primary infection in the cerebrum is obtained, it is only with the greatest difficulty that the virus can be carried on in series by brain inoculations alone. The cellular infiltration is more meningeal than perivascular and contains many large mononuclear cells; on the other hand, in *encephalitis lethargica* the cellular infiltration consists mainly of lymphocytes and glial cells.

CONCLUSIONS.

From the above observations it would appear that rabbit inoculations are of the greatest value in confirming a diagnosis of *encephalitis lethargica.*

Emulsions made of the basal nuclei give in most cases a positive result in the rabbit, while cerebrospinal fluid was only positive in one out of four cases.

It seems probable, from the findings of Kling, that a higher percentage of positive findings might be obtained with cerebrospinal fluid if the rabbits which show no signs after inoculation were killed 10 to 20 days later and the brain examined histologically.

In conclusion, it is necessary to impress on the observer that the important features of the lesion are (1) the site, (2) the nature of the cells, and (3) the presence of small foci of cells in the brain substance.

REFERENCES.

Kling, C., Davide, H., and Liljenquist.—(1921) *Compt. rend. Soc. de biol.*, **85**, 823.
Levaditi, C., and Nicolau, S.—(1921) *Compt. rend. Acad. des sci.*, **173**, 870.
Loewe, L., Hirshfeld, S., and Strauss, I.—(1919) *J. Infect. Dis.*, **25**, 378.
Loewe, L., and Strauss, I.—(1920) *J. Amer. Med. Assoc.*, **74**, 1373.
McIntosh, J.—(1918) *Rep. Local Government Board on Public Health and Med. Subjects*, No. 121, London (H.M. Stationery Office), p. 57.—(1920) *Brit. J. Exper. Pathol.*, **1**, 257.
Moussu, R.—(1922) *Rec. méd. vét.*, **98**, 499.
Oliver, J.—(1922) *J. Infect. Dis.*, **30**, 91.

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OF

EXPERIMENTAL PATHOLOGY

ON THE BEHAVIOUR OF PLATELETS IN VITAMIN A DEFICIENCY AND ON THE TECHNIQUE OF COUNTING THEM.

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In a series of papers we have shown (1921, 1922) that there is an essential similarity between the effects of radiations from X rays and radium on the one hand, and, on the other, of submitting animals to a diet deficient in vitamins. Thus a diet free from vitamin B produces atrophy of the lymphoid tissue throughout the body and a lymphopenia, just as radiations do. This is associated in both cases with marasmus, fall of temperature and eventually death in the absence of infections or other obvious lesions. A diet free from vitamin A was found to produce a thrombopenia, and when as the result of this finding the behaviour of blood-platelets in radiated rats was studied, it was found that the same lesion of thrombopenia could be produced if a sufficiently large dose of radium was applied. This thrombopenia was associated with the same general effects in both conditions, namely, an anæmia and a diminished resistance to infection. Recently yet another lesion has been found to be present in both conditions, namely, an atrophy of the intestinal epithelium. This will be dealt with in detail in separate communications. It is sufficient to state here that probably this lesion is the primary lesion which leads to invasion of the blood-stream by the intestinal bacteria, which are there agglutinated by the blood-platelets. This, by acting as a constant drain on the blood-platelets, exhausts their supply, and may thus account for the thrombopenia. In any case whatever the explanation may be, the generalisation that vitamin deficiency and radiations produce the same general effects has so far proved to hold good.

The occurrence of a thrombopenia after radiation has been confirmed by the independent work of Fabricius-Möller (1922), who studied the effects of X rays on guinea-pigs. He obtained a degree of thrombopenia even more profound than we could produce in rats with radium, and found that this intense thrombopenia was accompanied by hæmorrhages in different organs. And Lacassagne and his collaborators (1922) produced in newborn rabbits a condition of purpura hæmorrhagica with almost complete disappearance of platelets by applying X rays to the uterus of the pregnant mother shortly before birth.

The behaviour of the blood-platelets in vitamin A deficiency has been studied recently by Bedson and Zilva (1923) in rats. They conclude from their observations that there is a slight diminution of the blood-platelets in that condition. That diminution is, however, much less than the thrombopenia obtained by us. Thus while the diminution in our advanced cases was never less than 50 per cent. of the normal number and sometimes exceeded 75 per cent., Bedson and Zilva find that the diminution in their rats even when they exhibit xerophthalmia amounts maximally only to 20 per cent. In absolute figures, our normal rats have an average count of about 800,000 platelets per c.mm., and this falls in vitamin A deficiency to 400,000, 300,000 or even 200,000. The counts of Bedson and Zilva give for normal rats roughly 1,000,000 per c.mm., and for vitamin A deficiency rats a maximal fall to only 800,000. Bedson and Zilva state that they are unable to account for the discrepancy between our figures and theirs. They do not disclose the fact that in a small number of animals comparative counts were made by them and by us on the same animal. In normal rats these counts gave a reasonably close agreement. But in rats suffering from vitamin A deficiency the number of platelets found by Bedson and Zilva widely exceeded our counts, sometimes by nearly 100 per cent. And while our counts always indicated the presence of a marked thrombopenia, even in Bedson and Zilva's rats, their counts indicated only a slight thrombopenia or no thrombopenia at all, even in our rats. These facts are of importance, for they prove that the discrepancy must be due to differences in technique. It definitely eliminates the possibility that thrombopenia is an accidental phenomenon which may occur occasionally.

We shall demonstrate in this paper that Bedson and Zilva on their own showing used such an optical technique as would make an accurate counting of blood-platelets impossible, especially in such pathological conditions as vitamin A deficiency, where the blood contains a number of bodies similar in size to blood-platelets, but obviously different if examined with a microscope giving the highest possible resolution.

Owing to faulty microscopic technique errors have been made in the past by grouping together as "blood-platelets" all small bodies floating in plasma and being neither red corpuscles nor leucocytes. Thus faulty microscopic technique is responsible for the extraordinary mistake of denying altogether the existence of blood-platelets as preformed living elements of the blood. Some of the older histologists who carefully studied the behaviour and structure of blood-platelets in great detail have pointed out the error of confusing platelets with other bodies, and they insisted that blood-platelets must be identified by their characteristic structure. Under a good lens-system living unstained rat platelets appear in front view as circular, sharply outlined discs, having an average diameter of 2 to 3μ, and containing minute highly refractile granules grouped towards the centre. Seen edgeways a platelet is a very narrow concavo-convex disc with a slight thickening in the centre. In vitamin A deficiency the blood is full of bodies which do not have the structure of blood-platelets. Some of these bodies can be recognised as bacteria, others are undefinable and may represent lipoid globules or protoplasmic *débris*. This greatly adds to the difficulty of counting in that condition because every blood-platelet has to be identified by its structure. Since details of structure of blood-platelets are very near the limit of microscopic vision and have to be recognised in the living unstained cell, their identification requires the highest possible power of resolution a microscope can give. We find no mention of this difficulty of distinguishing blood-platelets from other bodies in the paper by Bedson and Zilva, and it is in fact impossible to do so with any degree of certainty with the optical arrangement used by these authors. We can say, however, that in the two rats which we received from them such bodies were present in large numbers. Since this question of microscopic optics appears to be so little appreciated, it is necessary to go into the matter more fully.

TABLE.

No. of rat.	Number of red cells.	Number of platelets.	Duration of vitamin A deficiency.	Growth.	Eye symptoms.	
442	9,880,000	340,000	8 weeks.	Slowed	None	
443	8,760,000	415,000	8 ,,	,,	,,	
444	7,480,000	240,000	12 ,,	Arrested	,,	
445	6,440,000	250,000	4 ,,	,,	Xeropthalmia	
446	10,040,000	1,070,000	Before deficiency	Normal	—	Exposure to mercury vapour lamp from 1st to 9th week.
	8,080,000	705,000	4 weeks	Slowed	None	
	8,920,000	706,000	7 ,,	,,	,,	
	7,400,000	297,000	16 ,,	Arrested	,,	
447	9,400,000	610,000	1 week	Normal	,,	Mercury vapour lamp from 4th to 7th week.
	7,720,000	380,000	4 weeks	Arrested	,,	
	9,040,000	460,000	7 ,,	,,	Xeropthalmia	
448	9,040,000	650,000	1 week	,,	None	
	7,320,000	575,000	4 weeks	,,	,,	
529	9,400,000	610,000	2 ,,	,,	,,	
531	8,920,000	890,000	1 week	Normal	,,	
	8,800,000	546,000	7 weeks	Arrested	,,	

As regards the phenomenon itself which is in question, we have since the publication of our first paper made further observations on the number of platelets in rats on a diet deficient in vitamin A in order to study the effect of light on the organism. As the following table shows, we never failed to obtain a deep thrombopenia corresponding in degree to that recorded in our first paper. We stated there that in our stock of rats the normal count lies between 9 million and 10 million red cells and between 700,000 and 900,000 platelets. It should be noted that most of the counts given in this paper were made while the animals were still free from obvious infections and had not yet reached the final stage of vitamin A deficiency, because it was desired to study the effect of light on the thrombopenia in different stages of the deficiency. Two rats are included in this table which had been treated with ultra-violet light. No. 446 received light throughout the first nine weeks of the experiments, and shows a greatly delayed thrombopenia as compared with rats Nos. 447 and 448 of the same litter. Rat 447 received treatment with ultra-violet light after a deep thrombopenia had developed, with the result that the fall in the number of platelets was arrested. We intend to publish detailed observations on the action of light in a separate paper.

THE NECESSITY FOR USING A PERFECT OPTICAL SYSTEM IN THE COUNTING OF PLATELETS.

In ordinary histological or bacteriological work differentiation of structure is obtained by means of staining, which reveals the various cell structures by colour contrast. For this purpose the old-fashioned Abbe condenser generally suffices, although every scientific microscopist knows that it is defective in many respects, and that in particular it is incapable of giving perfect resolution, no matter how perfect the objective is. It has, however, the advantage of being fool-proof, and enables the routine worker who, as Nelson has pointed out, has no knowledge of the use of a proper aplanatic cone in his illuminating system,* or of the necessity of tube length correction, to obtain reasonably good results. But when one examines living unstained cells—as is necessary in the counting of platelets—details of structure can be recognised only through difference in the refractive index of the different cell constituents. This requires the highest resolution of which the microscope is capable, especially when one is dealing with such minute structures as the granules in platelets, which are near the limit of microscopic vision. Then the condensing system becomes all-important.

It must be clearly understood that resolution and magnification are two entirely different things, and that no amount of magnification can compensate for imperfect resolution. This elementary principle of microscopic optics is not acted upon by Bedson and Zilva, for when they wish to improve their optical system they substitute a 2 mm. oil-immersion lens and a high-power ocular for a 4 mm. dry lens and a low-power ocular, while retaining their Abbe condenser. By doing so they merely magnify an imperfectly resolved image, but, as will be shown below, they do not to any extent improve the resolution of the image. Resolution depends on the optical angle of the cone of light illuminating the object and the apical angle of the cone of light entering the objective. The wider the angles the greater the resolution. A measure of the width of these angles is given by the "numerical aperture" (N.A.) of the condenser and of the objective. In scientific microscopy the resolving power of any optical system is therefore expressed by the mean of the actual working N.A. of the condenser and of the objective. We say advisedly "the actual working N.A.," because the nominal N.A. of the condenser given in the catalogues does not necessarily represent the actual working N.A. Bedson and Zilva in describing their technique state: "An Abbe condenser (N.A. 1·3) was used." Now the Abbe condenser transmits a cone of light having a N.A. of 1·3 only when a layer of oil is interposed between condenser and the slide. But since, as Dallinger pointed out, less than half of that cone is aplanatic, only the aplanatic portion of the cone takes part in the formation of the image. The utilised fraction of the cone of light transmitted by an Abbe condenser is therefore reduced from 1·3 to 0·45, which makes this condenser useless for critical microscopy. Thus a 2 mm. lens of N.A. = 1·30 with an Abbe condenser gives an effective N.A. of $\frac{0{\cdot}45 + 1{\cdot}30}{2} = 0{\cdot}87$. That is the optical system used by Bedson and Zilva. Our system consisted of 2 mm. apochromat objective of N.A. = 1·40 with a first-class apochromatic condenser which can transmit an aplanatic cone having the N.A. = 1·35. Our system gives therefore an effective N.A. of $\frac{1{\cdot}40 + 1{\cdot}35}{2} = 1{\cdot}37$—an increase in the resolving power of over 50 per cent. as compared with the imperfect system of Bedson and Zilva. In fact Bedson and Zilva could have obtained a better resolution with a dry apochromatic 4 mm. lens of N.A. = 0·95 if they had combined it with a first-class condenser transmitting an aplanatic cone of N.A. = 0·90. Such a system would give them a N.A. = 0·92 instead of the value of 0·87 for their optical system with an oil-immersion lens. To get better definition with the Abbe condenser they recommend closing down the iris diaphragm, thus still further reducing the width of the aplanatic cone upon which resolution depends.

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* All optical systems in the microscope are computed on the assumption that light enters the condenser with a plane homogeneous wave front and with the rays parallel to the optical axis of the system, that it is transformed by the condenser into light having a spherical homogeneous front with the object at the centre of the sphere, and is transformed again by the objective into light having a plane homogeneous wave front. The perfection of the image depends on the extent to which this condition is fulfilled.

In order to demonstrate the fallacies introduced by counting platelets with an improperly worked optical system, we have made counts of the blood-platelets of vitamin A deficient rats both with an improperly worked optical system and with a properly worked system. The latter consisted of a 2 mm. apochromat objective, N.A. = 1·40, with a holoscopic oil-immersion condenser having a nominal N.A. = 1·40 and giving an actual cone of N.A. = 1·35. The imperfect system consisted of a 2 mm. semi-apochromatic oil-immersion objective, N.A. = 1·30, and an Abbe condenser, and corresponds to the system used by Bedson and Zilva.

The following figures were obtained:

	Perfect optical system. N.A. = 1·37.	Imperfect optical system. N.A. = 0·87, as used by Bedson and Zilva.
Rat 1	250,000	610,000
Rat 2	575,000	869,000
Rat 3	380,000	711,000

With the imperfect optical system as used by Bedson and Zilva the figures are always higher, and in fact approximate to those given by these authors, and if we had used such a defective system we would also have failed to detect the intense thrombopenia which actually exists. We had the same experience when in a few cases counts were made by us on rats in A deficiency which had been counted previously by Bedson and Zilva, or when, conversely, Bedson and Zilva counted rats in A deficiency which had been counted previously by us. In every case the counts by Bedson and Zilva were much higher than our counts—sometimes twice as high. With the blood of normal animals these discrepancies are not so marked, because normal blood contains few bodies which could be mistaken for platelets.

These facts speak for themselves so far as the results of Bedson and Zilva are concerned. Speaking generally, they mean that when an imperfect optical system is used in counting blood-platelets in vitamin A deficiency, a large number of bodies are counted as blood-platelets which, when viewed with a better optical system, are recognised as not being blood-platelets.

THE TECHNIQUE OF PLATELET COUNTING.

In view of the errors resulting from imperfect microscopic technique, it may be useful to give a more detailed account of the method of platelet counting than we gave in our first paper.

The necessary requisites are:

(1) A suitable diluting fluid.

(2) A Thoma-Zeiss hæmacytometer.

(3) A first-class microscope stand provided with a 2 mm. oil-immersion apochromat objective and an oil-immersion condenser, giving a solid cone of illumination equal to at least three-fourths of the N.A. of the objective.

(4) Suitable screens to give a ruling in order to facilitate counting.

The diluting fluid we have used and found suitable is Toisson's fluid. It must be freshly made and filtered before use. The rat is deeply anæsthetised with ether, the tail immersed for a few seconds in a vessel containing warm water at about 45° C. and then dried with cotton-wool. The tail is then completely immersed in a watch-glass or evaporating dish containing Toisson's fluid. It is important that the tail must be thoroughly wetted with Toisson's fluid. The tail is now cut under the fluid about 1 or 2 in. from the end and blood allowed to flow into the fluid. It is essential that a free flow of blood is obtained. After allowing the blood to flow freely for a few seconds, the end of the tail is quickly transferred to a watch-glass containing about 2 or 3 c.c. of Toisson's fluid, so that the end of the tail is completely immersed in the fluid. Blood is allowed to flow until a dilution suitable for counting is obtained. We have found it best to have a dilution so as to get about thirty red blood-cells in one field. After a little practice it can readily be seen when such a dilution is reached. The tail is then removed from the saline fluid, dried, and the red blood-cell pipette of the hæmacytometer filled in the usual way for a red-cell count.

The diluted blood is now thoroughly mixed with a platinum loop, and a standard drop placed on a glass slide previously cleaned with chromic acid and stored in alcohol. A No. 2 cover-glass, previously cleaned, is placed on the drop, which should spread out as a single cell-layer if slide and cover-glass have been properly cleaned. The preparation is now ringed round with paraffin wax and put aside to allow the blood-cells to settle. In the meantime a red cell-count is made in the usual way. When this has been completed the slide for the platelet-count is placed under the microscope.

For the reasons given in the preceding paper the optical system used must fulfil certain conditions. The condenser must be an oil-immersion condenser, giving a cone proportionate to the N.A. of the objective, so that the cone of illumination used is at least three-fourths of the N.A. of the objective. This can be done with any of the modern oil-immersion condensers. The condenser must be accurately focussed and centred, so that the image of the illuminant is seen in the same plane as the object. The tube-length must be accurately adjusted. The eye-piece is chosen so as to give a magnification of about 1200 diameters.

In order to facilitate the counting some form of ruling is necessary. This may consist of a glass circle ruled in millimetre squares, which is dropped into the ocular. Or a modification of the "ghost micrometer," as devised by Prof. Dixon, F.R.S., may be employed, and has been found very convenient.* It consists of a glass slide ruled into square millimetres and fixed in front of the illuminant in the plane of the lamp iris diaphragm. This slide now takes the place of the source of the light and an image of the squares is found focussed on the object. As already stated, the most suitable dilution for counting is one where about thirty red cells and in a normal rat about three platelets are seen in one field. The number of red cells in the various fields is now counted along with the platelets, and thus the ratio of red cells to platelets is obtained. Each individual platelet must be identified by its structure. The distribution of the red cells in different fields and the proportion of red cells to platelets in each field should be even. If the proportion is very uneven another preparation should be made.

With a good preparation it is sufficient to count thirty to forty platelets. This involves the counting of 300 to 400 red cells in normal rats and of 600 to 800 red cells in vitamin A deficient rats. The statement of Bedson and Zilva that they counted 100 platelets must be viewed in the light of the demonstration given in the preceding section, that in the vitamin-deficient rats more than half the bodies which with a defective optical system are apt to be counted as platelets are not blood-platelets at all.

It may be added that in our experiments the factor of personal bias was completely excluded. The person who counted the platelets did not know what the condition of the animal was, and as a rule animals in a state of vitamin deficiency and normal animals or animals recovering from a state of vitamin A deficiency were subjected to examination at the same time.

SUMMARY.

The constant occurrence of a deep thrombopenia in vitamin A deficiency in rats has been confirmed by us by further observations. A detailed account is given of the technique of platelet counting, with special reference to the microscopic technique required to obtain reliable results. It is shown that with a faulty optical system the platelet-count in vitamin A deficiency gives

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* Dixon's "ghost micrometer" is supplied by T. H. Mason, Dublin; our modification may be obtained from James Swift & Son, Tottenham Court Rd., London.

figures which greatly exceed, sometimes to the extent of 100 per cent., those obtained with a correct optical system giving the highest possible resolution This is due to the fact that in vitamin A deficiency a number of bodies other than platelets but of similar dimensions appear in the blood and are mistaken for platelets when a faulty microscopic technique is employed. The optical system used by Bedson and Zilva, who, in repeating our observations, found only a slight thrombopenia in vitamin A deficiency, is a faulty one, and the platelet counts obtained by them greatly exceed the true counts obtained with a correct optical system. That the results of Bedson and Zilva are due to their microscopic technique and not to any difference in the reaction of their rats to vitamin A deficiency follows from the results obtained when counts were made on the same animal in three cases of vitamin A deficiency by them and by us. In every case their figures greatly exceeded ours.

REFERENCES.

Bedson, S. P., and Zilva, S. S.—(1923) *Brit. J. Exp. Pathol.*, **4**, 5.

Cramer, W., Drew, A. H., and Mottram, J. C.—(1921) *Lancet*, **1**, 963.—(1922) *ibid.*, **2**, 1202.—(1922) *Proc. Roy. Soc.*, **B**, **93**, 449.

Fabricius-Möller, J.—(1922) *Compt. Rend. Soc. de Biol.*, **87**, 759 ; also Monograph in Danish, Copenhagen, 1922.

Lacassagne, A., Lavedan, J., and de Léobardy, J.—(1922) *Compt. Rend. Soc. de Biol.*, **86**, 668.

A SUGGESTED SUBSTITUTE FOR CANADA BALSAM AS A MOUNTING MEDIUM.*

H. W. SOUTHGATE, M.B., B.Sc.

From the University, Sheffield.

Received for publication March 6th, 1923.

The effect of Canada balsam on the more delicate stains is known only too well to those interested in histology. In my opinion this is due mainly to the 63 per cent. of higher organic acids contained in this balsam.

In 1920 and the early part of 1921 efforts were made, but in vain, to obtain a neutralised non-crystalline solution of Canada balsam in xylol, by neutralising with various inorganic and organic bases.

The literature concerning other oleoresins was therefore consulted with a view to finding a substitute for Canada balsam, and it was found that a species of Elemi—that from Yucatan—contained no organic acids, but 60–70 per cent. neutral amorphous resenes besides volatile oil and amyrin. Through the offices of a mutual friend, Dr. Tschirch sent me the remainder of the original sample of Yucatan Elemi, his analysis of which had drawn my attention to its possibilities as a substitute for Canada balsam.

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* This note is an abstract of a fuller paper to appear in the *Journal of the Royal Microscopical Society*, and is published through the courtesy of the Editors of the latter.

The refractive index of a 60 per cent. solution of some of this in benzene was compared with that of a similar strength solution of natural (*i. e.* undried) Canada balsam in the same solvent, and gave—

60 per cent. Elemi solution	1·5146
60 ,, Canada balsam	1·5073

for the hydrogen red line.

Solutions of this Elemi also dried and set quickly.

Liver sections from a case of malignant malaria were stained with Giemsa and put up in a solution of the oleoresin in xylol. The colours were maintained but a difficulty rapidly appeared, *i. e.* the crystallising out of the amyrin compounds under the cover-slip. Efforts were made to remove these compounds by recognised methods but I was not able to remove sufficient to prevent some crystallisation taking place, and the investigation was discontinued in the summer of 1921.

Recently, however, I brought the possibility of this Elemi (provided a more complete removal of the amyrins could be effected) to the notice of a friend (Mr. P. P. Laidlaw), and he informs me that the following method removes some 25 per cent. of crystalline compounds (a bigger yield than I obtained), and that some slides mounted now for over three months in this medium show no trace of crystallisation, and, it is presumed, will not now.

He removes the amyrins by the following simple method:

200 grams of crude hard Yucatan Elemi are digested in the cold with 200 c.c. of industrial spirit (not methylated spirit) till all the lumps are broken up. The resene and the essential oil dissolve readily; the bulk of the amyrin crystals and the *débris* remain suspended, forming a mixture of creamy appearance. The amyrin crystals and the *débris* are filtered off at the water-pump, the filter cake being well pressed out. The filtrate is then evaporated down in the steam oven in shallow dishes, and when dry dissolved in *dry* xylol to the desired consistency for mounting.

Yucatan Elemi with the amyrins removed by the above method is therefore recommended as a substitute for Canada balsam as a mounting medium for the following reasons:

(1) It contains no organic acids but consists practically of neutral resenes.

(2) Complete solubility in alcohol, xylol, benzene, etc.

(3) A comparable refractive index.

(4) Its good drying and setting properties.

(5) Some sections stained with Giemsa's stain two years previously have the colours still unimpaired, mounted in this medium.

(6) Its relative cheapness: the crude mountant can be purchased from The British Drug Houses Ltd. for about 2*s.* 6*d.* a lb., and this should yield about 60 per cent. of neutral resene.

Finally it may be stated that Mr. C. Dobell reports very favourably on this Elemi solution in dry xylol as a mountant for the Romanowsky stains, methylene blue, etc. Methylblau, however, he informs me, is an exception.

My indebtedness to Mr. P. P. Laidlaw is obvious, and I would express my thanks to Mr. C. Dobell also for his kindly interest and criticisms.

GROWTH AND DIFFERENTIATION IN TISSUE CULTURES.

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Imperial Cancer Research Fund, London.

Received for publication March 6th, 1923.

THE study of pure cultures of cells *in vitro* permits an insight into the relations of cells of different kinds to each other, and leads to an analysis of the factors which determine their behaviour in the body with the ultimate possibility of the experimental control of the processes involved. Differentiation is one of the most obvious of these processes, and the observations to be described here indicate some of the factors which influence it, and reveal its reversible character. In an investigation of this kind technical details inevitably play an important part, and it will be necessary to describe the modifications which have been adopted as a result of extended trials since the publication of the previous communication in this Journal (Drew, 1922).

It has been shown already that cultures of both embryonic and adult tissues as well as tumours may be grown in a saline medium for an indefinite period so long as an embryonic extract is present as well. Growth does not occur without such an extract in the case of adult tissues, whilst in tumours and embryonic tissues generally a temporary growth takes place. The substance or substances in embryo extract responsible for maintaining growth are unknown, but the activity diminishes on keeping in the ice safe (4°–8° C.), and in two weeks is no longer of usable strength. They are destroyed by heating at 60° C. for 15 minutes. Filtration through a Chamberland F candle definitely reduces the strength of the extracts; filtration through a Berkefeld candle, however, leaves this property unimpaired, and gives a clear, nearly colourless sterile filtrate which may be kept for a much greater period without undergoing change. The method of preparation is as follows: Young rat or mouse embryos are cut up into coarse pieces with scissors in a sterile Petri dish. The fragments are thoroughly washed with several changes of the saline culture medium to remove traces of blood and are then finely minced. (In the preparation of all tissues for *in vitro* culture, blood must be got rid of as completely as possible, as if present in more than traces, it appears to inhibit growth.) The pulp so obtained is transferred to a large sterile tube and is frozen and thawed three times to disintegrate the cells and liberate the juices. An equal volume of the saline fluid is added to the emulsion, and is incubated for an hour at 37° C., the mixture being shaken at intervals. At the end of an hour the tube is centrifuged at high speed and the supernatant fluid pipetted off. It is then filtered through a Berkefeld filter and is stored in tubes in the ice chest.

It has been found best to use a final fluid made up as follows:

Saline solution	3 parts.
Embryo extract . . .	1 part.
Rat serum	1 ,,

The addition of the rat serum is not necessary for growth, which occurs perfectly well without it, but it makes the fluid more viscid, prevents the drop from spreading over the entire surface, and tends to stick the fragment of tissue to the cover-glass. To ensure adhesion the hanging-drop preparation is inverted and left in the incubator for about 6 hours, or indeed throughout incubation. It is only turned over when it is desired to examine the culture through the cover-glass, under the microscope.

Extract prepared in a similar manner from adult tissues gave little or no growth, but if very much stronger extracts were made by reducing the volume of saline added to about one-tenth they were found to be capable of replacing embryo extract to a certain extent. Such extracts were more potent when prepared from young rats or mice, indeed extracts from old animals appeared to exert an inhibitory effect, inducing early degeneration in the young growing cells. This phenomenon has already been noticed by Carrel and Ebeling (1921) to occur in plasma cultures, the plasma of an old animal retarding growth. Tumour extracts were found to be somewhat erratic, but could replace embryo extract with fair success, if used in sufficient concentration, to maintain growth in normal tissues. When tested with cultures of tumours they generally proved unsatisfactory, inducing early degeneration. This effect is probably due to toxic bodies, as it has already been shown that cultures of actively growing tumour cells appear to produce a substance capable of inhibiting growth in tumour cultures.* A yeast extract, rich in vitamin B, was tried but was found to be perfectly inert, and could in no way replace embryo extract. A pea flour extract likewise was quite useless. Apparently both adult tissue extracts and tumour extracts contain the substance or substances present in embryo extract, but in the case of tumours this is masked by the toxic bodies, whilst old tissues contain an inhibitory substance.

I have already shown that differentiation of the cells in a culture depends upon the presence or absence of connective tissue, but the best demonstration of this fact is seen when one grows pure cultures of cells, *e. g.* kidney or skin, and then adds connective-tissue elements. Pure cultures may be obtained with little difficulty by the mercury drop method used for obtaining single cell bacterial cultures. A fragment of embryonic kidney is planted out in the fluid medium and incubated at 37° C. The fragment is examined daily, and whether it shows commencing growth or not, fresh medium is supplied each day. At the end of four or five days, sometimes earlier, the kidney cells will be found growing out as elongated processes which ultimately form a sheet of undifferentiated tissue. The processes in many cases are quite free from accompanying connective tissue. A growth is selected with processes free from other cells, and a globule of mercury is carefully placed on the slide and pushed over a projecting process of parenchyma cells so as to cover it. The slide is then removed from the microscope and is exposed to a quartz mercury vapour lamp for ten minutes, care being taken to avoid heating. Fresh culture medium is added and the slide returned to the incubator. The cells unshielded by the mercury globule are killed and growth now proceeds from the isolated process. The culture is continued till a large sheet of undifferentiated kidney cells is obtained (Fig. 1, and, in the case of mammary tissue, Fig. 2), when

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* The growth-activating substance from autolysed normal tissues, which is mentioned on a subsequent page, has a similar action when applied continuously to growing normal tissues, but it seems probable that the toxic action of extracts of tumour cultures is not identical.

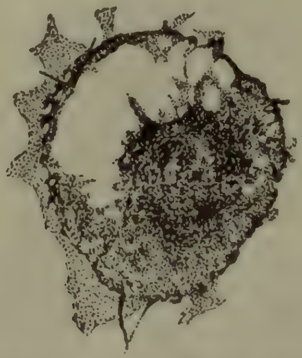

Fig. 4.—Mouse carcinoma 206 in culture in saline medium + embryonic extract. Low power view. See Plate-fig. 4*a*.

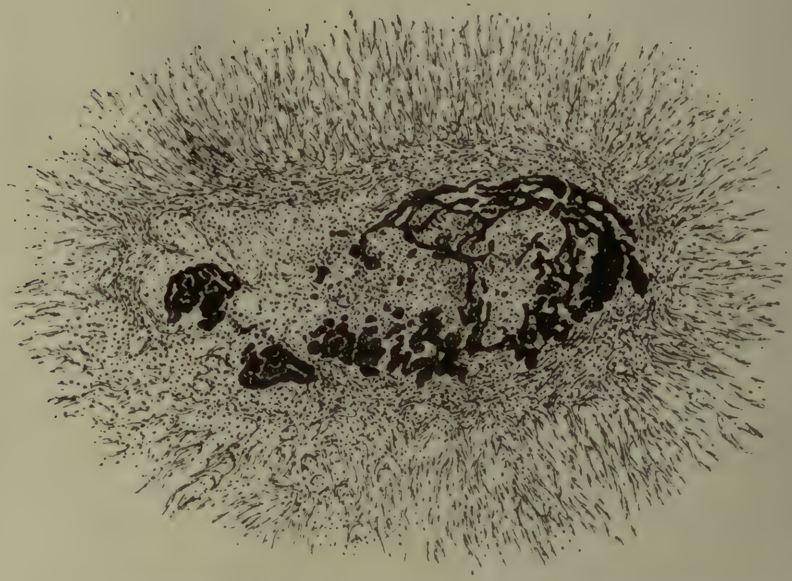

Fig. 5.—Mouse carcinoma 206 in culture with connective-tissue elements. Note acinus formation. The whole growth strongly resembles normal mouse mamma.

a small fragment of a connective-tissue culture is added and incubation continued. In a certain proportion of cases the connective tissue may fail to grow, but in those cultures where it does so differentiation sets in in about two to three days, and a culture is obtained containing free connective-tissue cells, undifferentiated kidney cells and kidney cells forming typical tubules (Figs. 3 and 3*a*). The same process is shown very well with tumours. For example, an alveolar mammary carcinoma, 206, tends in artificial culture to give pure parenchyma growths, as a sheet of undifferentiated cells (Figs. 4 and 4*a*), without having recourse to exposure to ultra-violet rays. When such a culture is obtained, either by aid of the ultra-violet light or from cultures growing pure from the start, connective-tissue cells may be added either from a culture or from a fresh piece of tissue planted direct. Fig. 5 illustrates the type of growth obtained. Where the tumour cells come into contact with the connective tissue they form acini, the whole culture strongly resembling normal mouse mamma (Figs. 6 and 7). This experiment shows that the undifferentiated condition of the parenchyma of this tumour is not an anaplasia in v. Hansemann's sense (*i. e.* a permanent irreversible loss of differentiation), but merely a temporary growth form which can differentiate out in a suitable environment. The connective-tissue elements may be derived from any organ. For example, connective tissue derived from heart will cause the formation of acini in a mammary carcinoma or the formation of tubules in cultures of kidney. That there exists a delicate balance between the two types of cells is clearly shown in continued culture, for if such a culture, say of kidney, which has had tubule formation induced by the addition of connective-tissue elements to it, be continued, it is found that gradually only one or other of the types of cell will persist, and ultimately either a culture of connective tissue or of pure kidney cells will be obtained again, when the cells grow as a sheet of undifferentiated tissue as before. In most cases the connective-tissue cells grow more rapidly than the parenchyma, and ultimately tend to overgrow it. In the case of mixed cultures of tumour and connective tissue artificially added, the tumour cells rapidly degenerate unless very frequent subculture is performed and one ultimately obtains a culture of connective tissue only. Cultures of carcinoma with added stroma exhibiting differentiation reproduce the original type of tumour on reinoculation into mice.

Attempts to inhibit the growth of tumours in mice by means of the toxic products elaborated *in vitro* have not proved successful. Whatever the toxic substance is that is formed in culture, it is apparently easily destroyed in the body and fails to influence the growth of the tumour. Cultures of normal tissues grown for many generations outside the body show no tendency to grow when inoculated into animals but rapidly disappear.

We have found it preferable when removing normal or malignant tissues for cultures to store them in the ice-chest when immediate culture is not possible. Such cold storage, provided the tissues are aseptic, appears to do no harm, growths having been obtained after as long a period as thirteen days. Comparatively slight alterations in the incubation temperature appear to have a considerable effect on the cultures. For example, heart cultures incubated at 40° C. give growths of connective-tissue cells only, the heart muscle cells showing fatty changes and rapidly dying out. Mammary carcinoma incubated at the same temperature gave pure connective-tissue growths after two generations, the tumour cells degenerating. Kidney, on the other hand, appeared to be little influenced by this temperature, the same proportion of kidney growths resulting as at 37° C. Tumours when grown on slides completely immersed in the culture medium are interesting from the fact that portions become detached and grow on distant parts of the slide forming what may be termed artificial metastases: such a growth is illustrated in Fig. 8.

An interesting fact is brought out in comparing the malignancy with the degree of growth *in vitro* of a series of tumours of varying degrees of malignancy, for example 37 S, 63, 206, Jensen, 630, 27. These are arranged in Table I in order of their rapidity of growth in the mouse. *In vitro* cultures of these tumours show growth which may be represented by the *plus* signs in the table:

TABLE I.

Rapidly growing spindle-cell sarcoma 37 S	+ + +
Rapidly growing alveolar mammary carcinoma 63	+ + +
Mammary carcinoma, rapidly growing, but with much absorption 206	+ +
Variably growing alveolar mammary carcinoma Jensen	+
Squamous cell carcinoma 630	slight + after lag
Adeno-carcinoma of mamma 27	,, ,,

Where growth is obtained at all from 630 and 27 it closely resembles that of adult tissues, being small in amount at first and always exhibiting the lag which seems characteristic of adult mouse and rat tissues.

The ease with which the several factors, which may be supposed to act in growth and repair, can be separately varied in *in vitro* culture has already been noted. Striking differences are produced in the cultures of adult tissues by adding to the culture medium an extract of autolysed adult tissue. Adult kidney was ground up with sterile sand and some of the saline medium; the resulting emulsion was incubated for one hour at 37° C., and was then filtered through a Berkefeld candle. Two series (A and B) of primary cultures of heart and kidney obtained from a full-grown mouse were put up in media having the following composition:

A.	Saline solution	3 parts.
	Embryo extract	2 ,,
	Rat serum	1 part.
B.	Saline solution	3 parts.
	Embryo extract	1 part.
	Autolysed kidney extract	1 ,,
	Rat serum	1 ,,

A dozen cultures of heart and kidney were put up in each of the solutions A and B, incubated at 37° C., and examined daily.

In the cultures without damaged tissue extract no growth occurred till the tenth day, when a mixed growth containing connective-tissue cells and kidney and heart cells respectively was obtained from 8 of the 12 cultures (Fig. 9).

Cultures in solution B showed connective-tissue growth within twenty-four hours in all cases, but only slight growth of the parenchyma took place till the second day, when all the cultures showed a mixed growth of both kidney and connective-tissue cells (Fig. 10). It will be seen that there appears to be some substance or substances in an extract of damaged tissue capable of accelerating the growth both of connective tissue and parenchyma. Heating to 80°C. for 15 minutes had no effect on the growth-promoting substances. A feature of the growth obtained by the addition of such autolysed extracts is its explosive character—that is to say, the original fragment may disappear and fragments of rapidly growing cells be found scattered over the slide. If such growths be subcultured into fresh medium containing autolysed extract little more growth is obtained as rapid degeneration sets in. If, however, the subculture is made with the ordinary culture fluid, vigorous growth will continue and the cultures may be maintained indefinitely.

It seemed likely that tumours might contain such a growth-accelerating substance ready formed, and to test this supposition extracts of tumours were prepared by grinding up tumour cells in a mortar cooled with ice. A small quantity of the saline medium was added and the mixture centrifuged at once without incubation. On testing such extracts it was found that a very potent growth-accelerating substance was present, giving as much growth with adult tissues as did an autolysed extract of normal tissues (Figs. 11, 12, 13). The tumours tested gave varying amounts of the activating substance. Thus both carcinoma 63 and sarcoma 37 yielded extracts of great potency, and both these tumours are of a high degree of malignancy and of very rapid growth (*cf.* Table I). Carcinoma 27, on the other hand, which is of low malignancy and slow growth, yielded only small traces of the activating substances, being in fact intermediate between the very malignant tumours and normal tissue.

The activating substances in autolysed extracts of normal adult tissues and their absence in embryonic tissue extracts shows that they are undoubtedly the same agents which are met with in the phenomena of repair. Their presence in cold extracts of the malignant tumours suggests that it is by means of a continuous production of these substances that tumour growth is maintained, and constitutes a new and very striking difference between tumours and normal tissues, the former containing the growth-stimulating factors, whilst the latter require to be severely damaged before they are produced. The fact that the highly malignant and rapidly-growing tumours contain a far greater amount of such growth-activating substances than do the slowly-growing and more benign, reveals a quantitative relation which has not before been demonstrated in the analysis of tumour growth.

SUMMARY.

The substances necessary for continuous growth which are present in extracts of embryonic tissues are able to pass through a Berkefeld filter. Adult tissues and tumours appear to contain these substances in lower

concentration. Tissues and tumours when grown free from connective-tissue elements grow as undifferentiated sheets. If connective tissue be added to such growths differentiation sets in. Autolysed extracts of normal adult tissues contain a growth-activating substance which when added to cultures of normal adult cells causes very rapid proliferation. Extracts of tumours, prepared in the cold and not subjected to autolysis, also contain this substance, the tumour cells appearing to be able to form it continuously.

REFERENCES.

Carrel, A., and Ebeling, A. H.—(1921) "Age and Multiplication of Fibroblasts," *J. Exp. Med.*, **34**, 600.

Drew, A. H.—(1922) "A Comparative Study of Normal and Malignant Tissues Grown in Artificial Culture," *Brit. J. Exp. Pathol.*, **3**, 20.

DESCRIPTION OF PLATES.

Fig. 1.—Four days' growth of embryonic mouse kidney in saline medium + embryonic extract, showing growth of parenchyma as undifferentiated sheet of cells.

Fig. 2.—Culture of normal pregnant mouse mamma in saline medium + embryonic extract. Ten days' growth, showing sheet growth of undifferentiated cells. Note formation of milk.

Figs. 3 and 3*a*.—Portion of culture of mouse kidney to which connective-tissue elements have been added. Note tubule formation. Four days' growth. Low and high power.

Fig. 4*a*.—Mouse carcinoma 206 in culture in saline medium + embryonic extract. High-power view. Note growth as undifferentiated sheet.

Fig. 6.—Mouse carcinoma 206 grown with embryonic rat heart. Five days' growth. Note formation of acini.

Fig. 7.—High-power view of portion of culture shown in fig. 6.

Fig. 8.—Culture of adult rat kidney in saline – embryonic medium with addition of tumour extract. Note expansive character of growth. Portions become detached and grow in various directions over the slide. Forty-eight hours' growth.

Fig. 9.—Culture of adult rat kidney in saline medium + embryonic extract. Eleven days' growth. Note almost entire absence of cell proliferation.

Fig. 10.—Culture of adult rat kidney in saline medium + embryonic extract with addition of autolysed tissue extract. Forty-eight hours' growth. Note extremely rapid proliferation of cells.

Fig. 11.—Culture of adult rat kidney in saline – embryonic medium with addition of extract from tumour 63 extracted in cold without autolysis. Forty-eight hours' growth. Note rapid growth similar to that obtained with the use of an autolysed normal tissue extract.

Fig. 12.—High-power view of portion of culture shown in fig. 11, showing perfectly normal kidney cells with mitochondria.

Fig. 13.—High-power view of portion of last culture, showing perfectly normal mitosis.

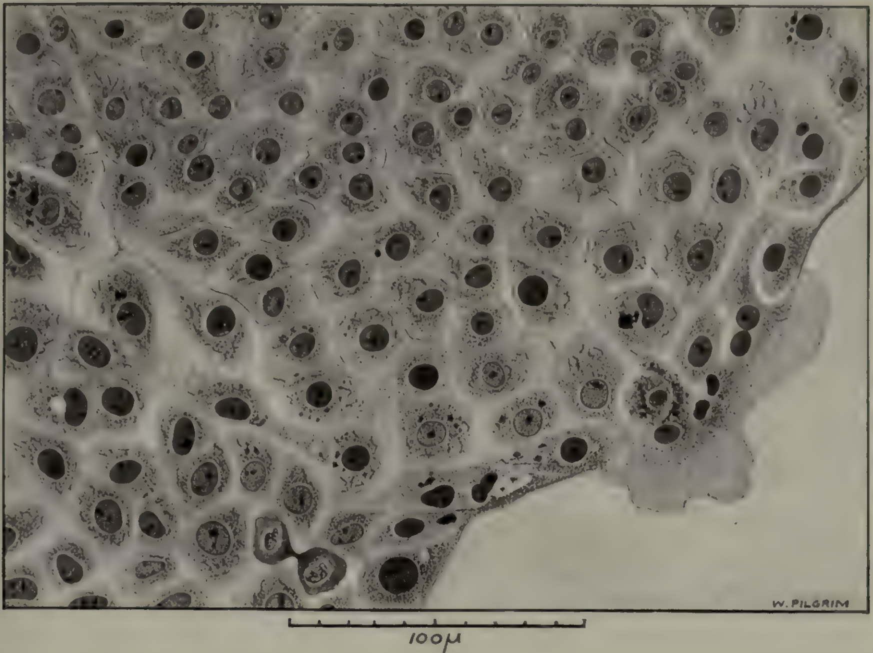

FIG. 1.

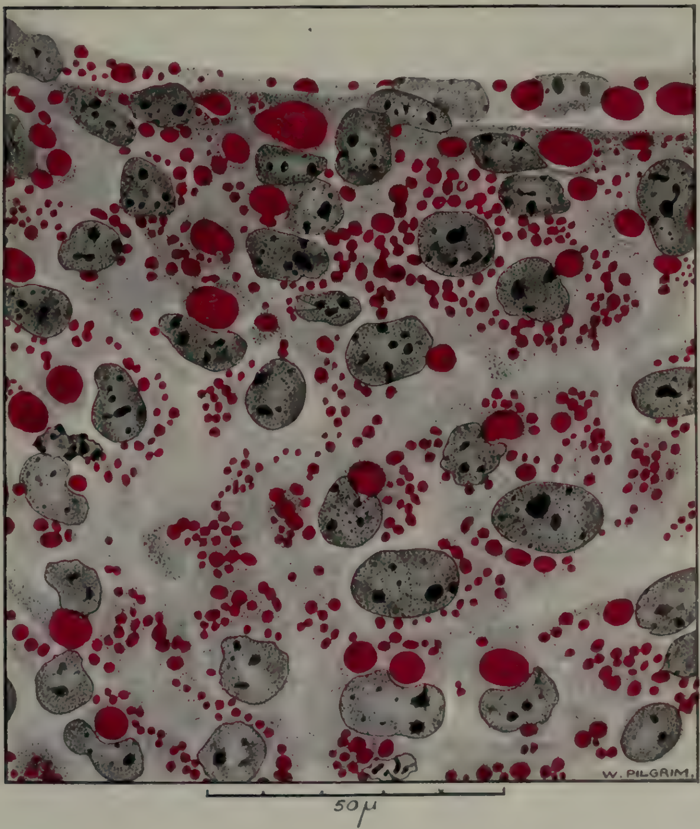

FIG. 2.

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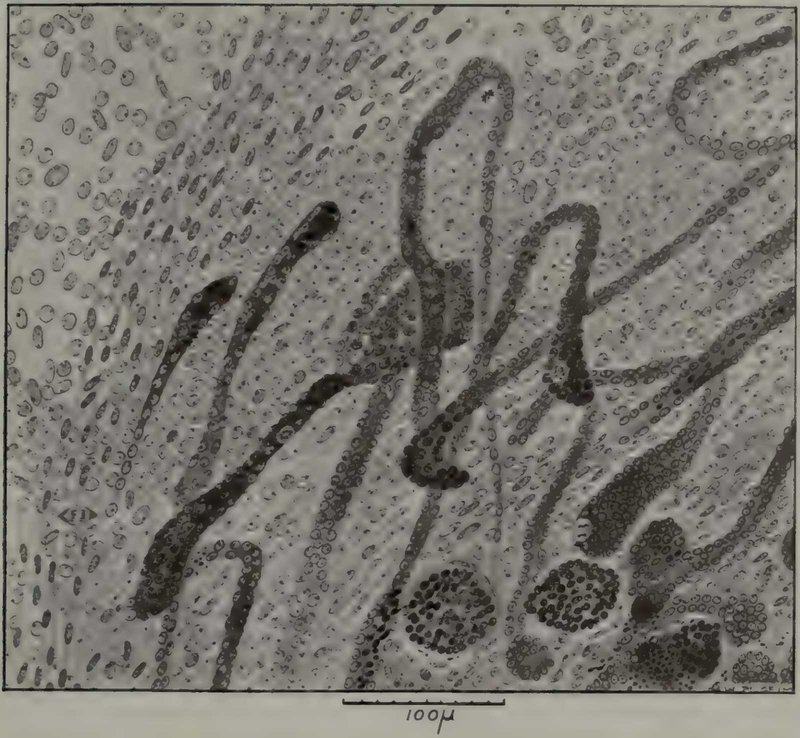

FIG. 3.

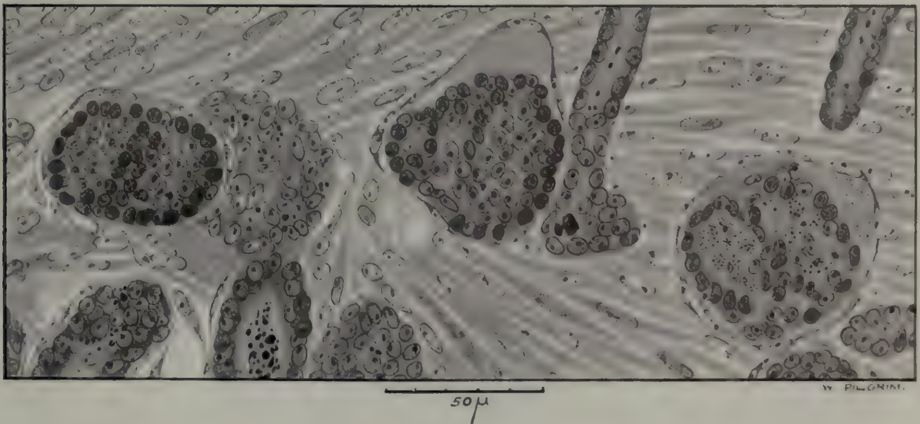

FIG. 3*a*.

Drew.

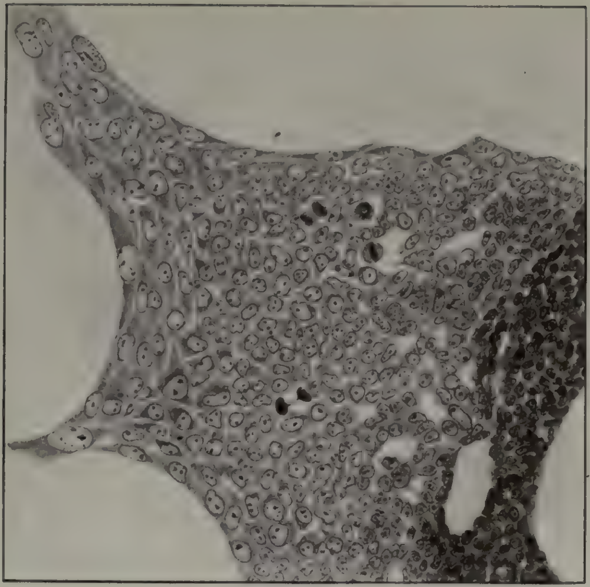

FIG. 4*a*.

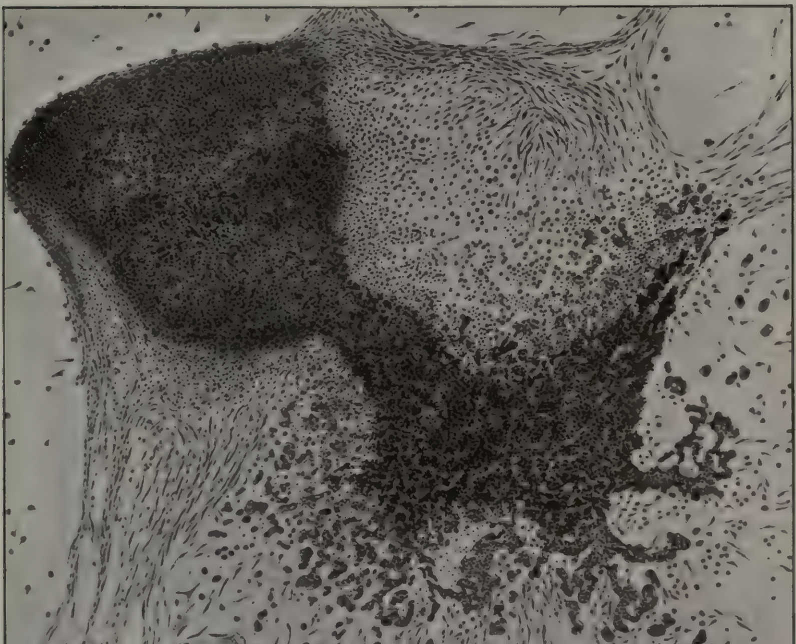

FIG. 6.

Drew.

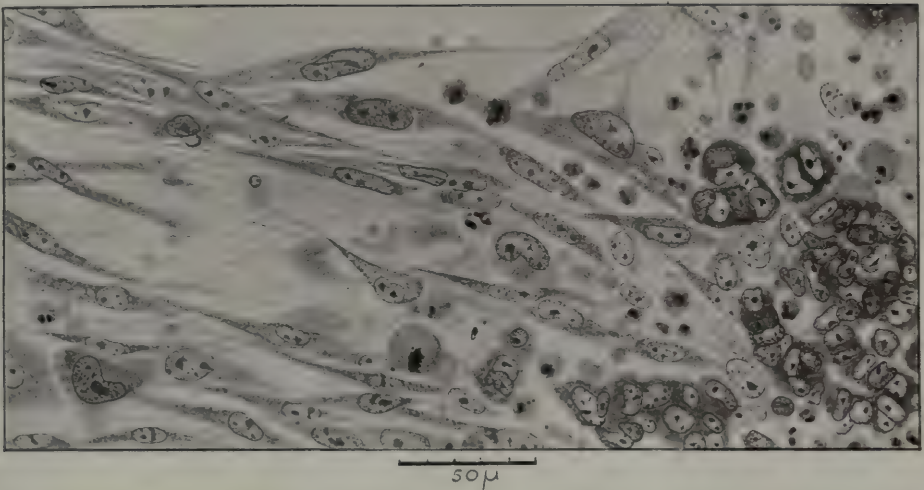

Fig. 7.

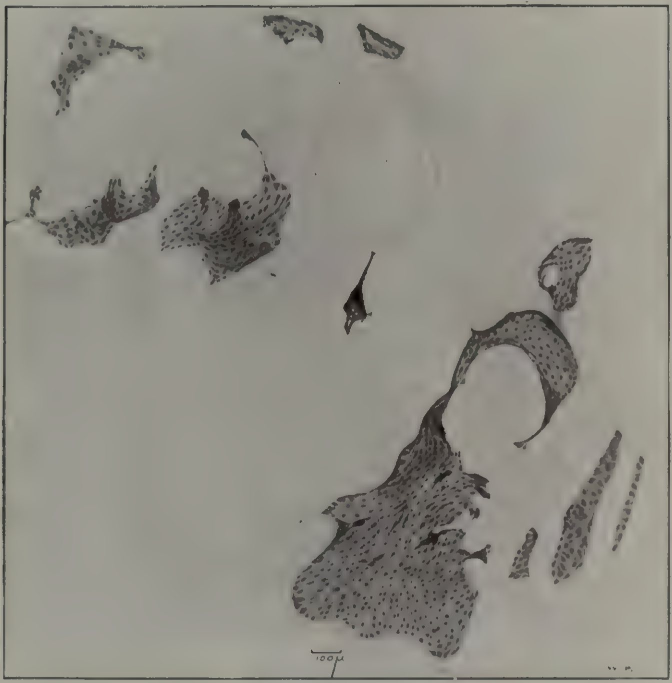

Fig. 8.

Drew.

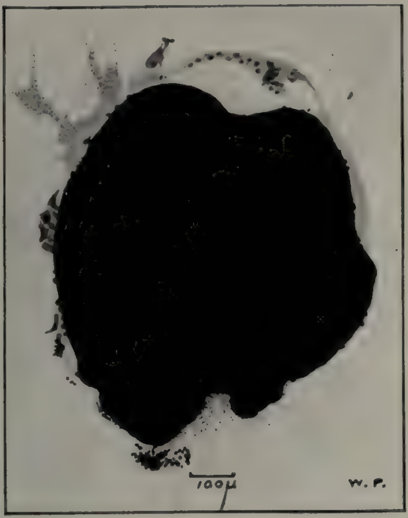

Fig. 9.

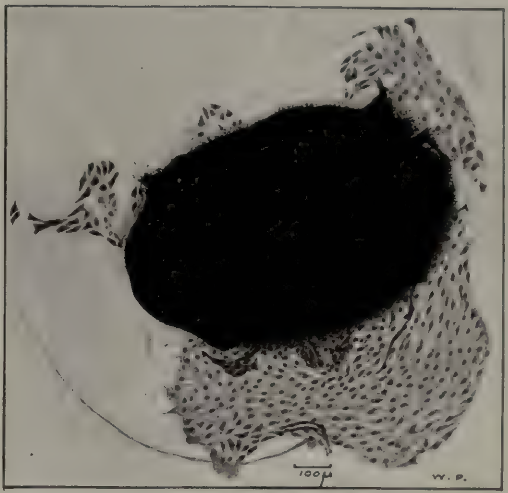

Fig. 11.

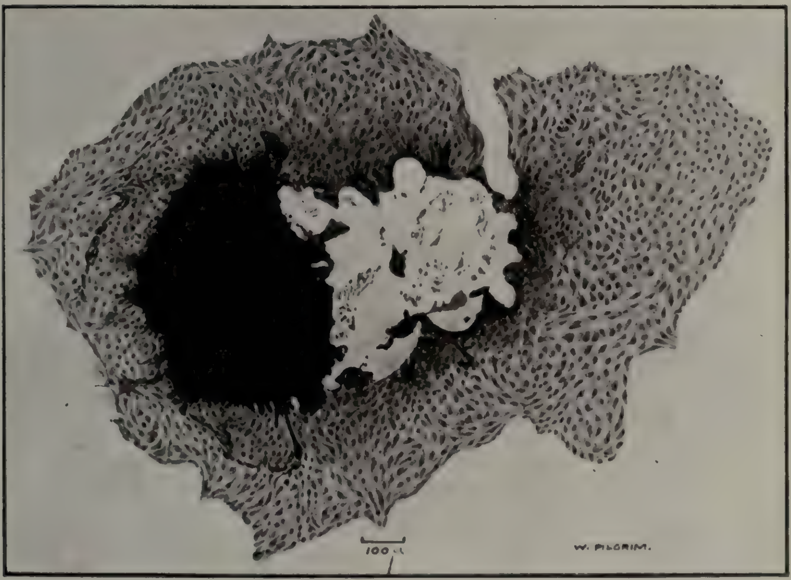

Fig. 10.

Drew.

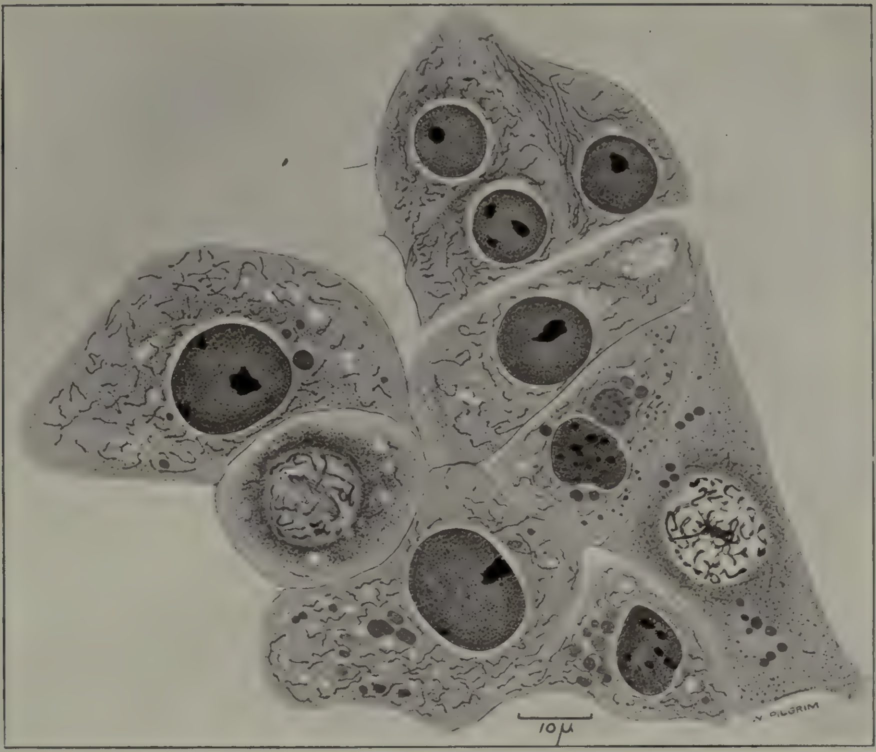

FIG. 12.

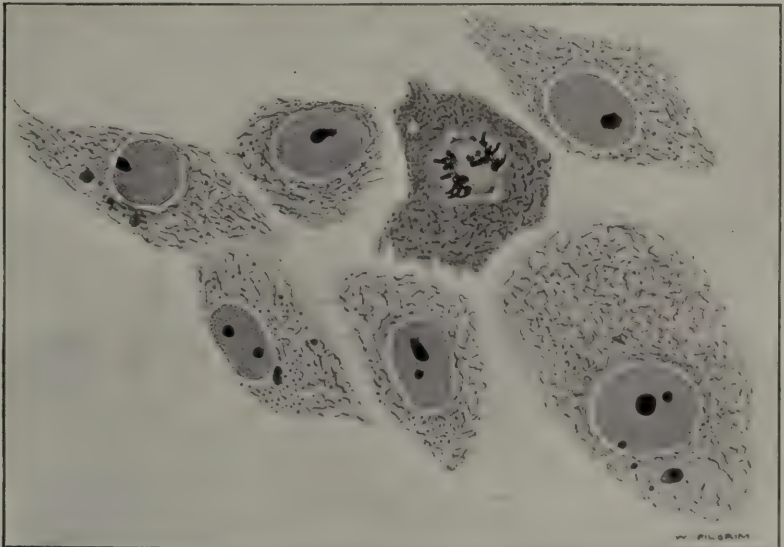

FIG. 13.

Drew.

ON THE MODE OF PRODUCTION OF BACTERIOPHAGE.

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The following experiments were undertaken with a view to discovering the conditions necessary for securing a bacteriophage for *B. coli* in its most potent form.

In an investigation into the rate of action of bacteriophage (Lepper, 1923) it had been found that the effect of the lytic substance is very rapid, a large proportion of the bacilli treated being killed in five minutes.

It was also found that guinea-pigs which had been injected intraperitoneally with a mixture of bacteriophage and a lethal dose of *B. coli* lived for several days, whereas the control guinea-pigs which had received the same dose of organisms without the lysin died in a few hours.

It seemed possible, therefore, that if a method could be found for producing bacteriophage for different strains of *B. coli*, beneficial results might be obtained by local treatment. In chronic pyelitis, for instance, an autogenous bacteriophage introduced into the pelvis of the kidney might be of therapeutic value.

Origin of the bacteriophage employed.—The bacteriophage which provided the material for investigation in these experiments was obtained from the urine of a woman suffering from bilateral pyelitis caused by *B. coli communis.*

Specimens of urine were passed through a Berkefeld filter, and varying amounts of the filtrate incubated in peptone water with the autogenous culture obtained from the same urine.

The third sample filtered yielded a positive result, as subcultures on agar made from the mixture after three hours' incubation showed a few large "nibbled" colonies only, growth inhibition being almost complete. After twenty-four hours' incubation subcultures from the mixture showed a good growth of the organism, but many typical "colonies of bacteriophage" or circular patches free from growth.

The strength of this bacteriophage has been considerably increased by passage with the autogenous organism.

The cultural characteristics of the B. coli *concerned.*—When first isolated this organism appeared to be a perfectly ordinary *B. coli*, but in the course of various experiments in which a peptone-water culture, to which no lysin had been added, was always put up as a control to the experimental tubes, it was found that every now and then subcultures from this control tube on agar showed a growth consisting solely of "nibbled" colonies.

This peculiarity of the culture was met with when plating experiments to test the strength of different bacteriophages were being made. On two occasions the control culture, to which no lysin had been added, after growing normally for some hours so that the peptone became definitely cloudy, was noticed at a later examination to be less cloudy, and was found to contain fewer bacilli per drop than it had done some hours previously.

The culture in fact behaved as a mixture of bacilli and bacteriophage such as d'Hérelle has described as occurring particularly in strains isolated from urine.

The culture when plated yields two types of colony, viz.:

(A) *Round type.*—These are the most numerous, are slightly opaque and raised.

(B) *Crescentic type.*—These are larger, rather transparent flat colonies with irregular edge.

The appearances obtained when the culture is treated with the bacteriophage.—Subcultures from a mixture of the bacillus and the bacteriophage will, in addition to round and crescentic colonies, show large raised opaque colonies which present one or more circular bites at the periphery. These are called "nibbled" colonies, and they appear when the culture has been treated with a strong filtrate. They have been found to be more resistant to the action of the lysin than the other types.

A fourth variety also appears, viz. the mucoid colony, which turns up as a resistant variety during the action of strong lysins, either by itself, or with the "nibbled" form. It is a raised transparent colony which grows rapidly at room temperature, and runs in a mucoid stream down the surface of the agar slope and forms a semi-fluid deposit at the bottom of the tube.

If subcultured and kept on the bench, the "mucoid" form maintains these characteristics, but in the incubator it reverts quickly to the "round" type.

Less active lysins produce the classical appearance of numerous circles free from growth.

Very weak lysins produce a growth which appears to be a mixture of opaque and transparent colonies, resulting in a very uneven-looking slope. This is called the "mixed" culture.

QUANTITATIVE METHODS FOR ESTIMATING THE STRENGTH OF BACTERIOPHAGE.

When a bacteriophage of moderate strength acts on a sensitive organism there is an immediate effect during which a certain number of the bacilli are killed, the number destroyed depending on the strength of the lysin, and a secondary effect in course of which the lysin is reproduced. The exact relation of the secondary to the primary effect has not been definitely worked out.

Of the quantitative methods which have been most used, that of d'Hérelle depends entirely on the secondary effect, that of Maitland (1922) and the somewhat similar method of Otto, Munter and Winkler (1922) aim at combining the results of both effects, while that of Appelmans (1921) depends on the primary effect, if the readings are made at the end of three hours, and on both effects if the tubes are incubated overnight.

None of these methods were selected for the following reasons:

(1) The existence of the zonal phenomenon, described by Ellis (1922), examples of which are given later in this paper, necessitates that to yield quantitative results, the number of organisms used in d'Hérelle's method should be standardised and the period of incubation fixed.

(2) The methods which are based on the appearances resulting when mixtures of bacteriophage and bacilli are grown on agar, are open to the objection that the personal factor must play a large part in assigning to any particular culture a quantitative value, the appearances being so varied that it is not easy to place them in order of magnitude and bacteriophage action. These methods may suffice for comparative results in the hands of an individual worker, but do not furnish a sufficiently accurate standard for quantitative results by different observers.

(3) Appelmans' dilution method requires to be supplemented by subculture. Certain dilutions of bacteriophage and bacilli may remain relatively clear as compared with the control culture to which no lysin has been added, and yet show numerous colonies if subcultured on agar.

As the object of these experiments was to obtain lysins which would have a marked immediate effect on the bacilli, *a method which would measure the actual lethal action was aimed at.*

Details of the method selected.—The method used was based on that employed at the Lister Institute for comparing the strength of disinfectants.

Dilutions of the bacteriophage to be tested are made in 4·9 c.c. peptone-water. To each of these, 0·1 c.c. of an emulsion of bacilli is added so that the total volume amounts to 5 c.c.

It has been found that a dose of organisms suitable for demonstrating the lethal effect of the lysins may be obtained in the following way:

A peptone-water culture is made overnight. In the morning 0·1 c.c. of this culture is delivered, by means of a standardised dropping pipette, into 4·9 c.c. peptone-water and the mixture incubated for three hours. This three-hours-old culture is diluted 1/10 with saline, and 0·1 c.c. of the resulting suspension added to each dilution of bacteriophage and to a control tube containing 4·9 c.c. of peptone-water only. An interval of one minute is made between the inoculation of each tube in order that sufficient time may be available when the subsequent subcultures are made.

Plates made from the control tube give a count varying between 1800 to 2200 colonies per drop (0·02 c.c.).

The tubes containing the lysins and bacilli are kept at room temperature and subcultured at minute intervals at the end of 4 hours and 24 hours, by delivering 0·01 c.c. from each tube into two tubes of lactose-litmus peptone-water, and 0·1 c.c. are also transferred to an agar slope.

These subcultures are incubated for 48 hours.

If no growth occurs in the lactose tubes then the bacilli must have been reduced to less than one per drop; if the bacteriophage has not been sufficiently active to produce this result the agar slopes are useful in showing evidence of bacteriophage action in a particular dilution.

By putting up tubes containing various amounts of carbolic acid and inoculating them at the same time with the same number of organisms, the activity of the bacteriophage can be compared with that of the chemical disinfectant.

EXPERIMENTS TO SHOW THE INCREASE OF BACTERIOPHAGE BY PASSAGE.

EXPERIMENT I.—*To study the effect on a heterogeneous culture.*

The various samples of bacteriophage used in these experiments were obtained by growing the bacillus isolated from a case of pyelitis in broth with some of the filtered urine from the same patient, specimen "E." The filtrate of this culture was called "Ea." This was used for a second passage, was filtered and called "Eb" and so on.

The action of these filtrates on a sensitive bacillus "Merrill" is shown in

Table I. The number of passage cultures which had taken place are given in the second column of the table. Unfortunately some of the samples had been used up in earlier experiments, so that it is not possible to give the complete series.

It will be seen that it is only after the fourth passage that a 1 in 10 dilution of the bacteriophage was able to reduce the bacilli from 1281 per drop (0·01 c.c.) to less than one per drop. The third sample, however, showed evidence of bacteriophage on the agar slope made from both dilutions at the end of two hours, well-marked circles being present. On counting these it was found that the slope from the 1/10 dilution showed 11 circles, while that from the 1/20 dilution showed 16.

In this experiment some of the dilutions were plated at the end of 2½ hours, the number of bacilli left per 1 c.c. being calculated in each case and displayed in column No. 6. In the early experiments plating was thus employed in order to control the results obtained in the fermentation tubes. The plates confirm the tubes except for sample 1/5000 of "Flask I."

TABLE I.—*To Show that Passage Increases the Activity of Bacteriophage.*

The experiment was carried out at 18° C., with a heterogeneous organism. 128,100 bacilli were present in 1 c.c. at beginning of experiment.

Filtration.	No. of passage.	Dilution.	0·01 c.c. into 2 lactose tubes.		Bacilli per 1 c.c. at 2½ hours.
			2 hours.	24 hours.	
E	None	1/5	+ +	+ +	*
		1/20	+ +	+ +	128,500
Ec	3rd	1/10	+ +	+ +	*
		1/20	+ +	+ +	95,500
Ed	4th	1/10	− −	− −	20
		1/50	+ +	+ +	450
Ee	5th	1/5	− −	− −	10
		1/10	− −	− −	...
		1/20	+ +	− +	200
Round flask	6th	1/10	+ +	− +	20
		1/50	+ +	− +	150
Flask I	9th	1/100	− −	− −	...
		1/500	− +	− +	130
		1/1000	− +	+ +	...
		1/5000	− +	+ +	950
Carbolic acid	—	1/200	− +	− −	...
		1/400	+ +	+ +	...

+ = Acid and gas in one lactose tube. + + = Acid and gas in both.
* Colonies too numerous.

Summary of Table I.—The table shows that the fourth, fifth and sixth filtrates are stronger than the third, while the ninth is considerably stronger than any of the others.

EXPERIMENT II.—*To study the effect on the autogenous culture.*

Having found a definite increase in strength of the bacteriophage as evidenced by its action on a heterogeneous culture it was of interest to find out—

(*a*) Whether the autogenous culture was more susceptible than the heterogeneous, and (*b*) whether there was any difference in the activity of the bacteriophage towards the two types of bacilli which could be isolated from the autogenous strain.

The results are shown in Table II.

TABLE II.—*To Show the Action of Various Samples of Bacteriophage on the Autogenous Strain.*

Experiment at room temperature.

Filtrate dilution.	No. of passage.	Type of bacillus.	No. of bacilli per drop (0·02 c.c.) at end of 2½ hrs.		0·01 c.c. into lactose—	
			Experimental.	Control.	4 hours.	24 hours.
E 1/10	None	Round	4345	3450	+ +	+ +
		Crescentic	3400	3838	+ +	+ +
Ec 1/10	3rd	Round	798	3450	— —	— +
		Crescentic	3750	3838	+ +	+ +
Ee 1/10	5th	Round	0·2	2170	— —	— —
1/100		,,	...	...	— —	— —
Round flask						
1/10	6th	Round	0	2170	— —	— —
1/100		,,	...	...	— —	— —
Flask I						
1/500	9th	Round	2	2170	— —	— —
1/1000		,,	...	...	—	— —
1/5000		,,	...	...	—	— —
Carbolic 1/250	...	Round	...	...	— +	— —
Acid 1/500	...	,,	...	...	+ +	+ +

Summary of Table II.—(1) It will be seen that the various samples of bacteriophage do act in higher dilutions on the autogenous strain. Filtrate "Ee" for instance entirely killed the autogenous culture in a dilution of 1/100, whereas a dilution of 1/20 of the same filtrate was not lethal for the heterogeneous strain.

The action of higher dilutions of the strongest bacteriophage, viz. that obtained after the ninth passage, is shown in Table III (see below). Its action on the autogenous strain is about ten times greater than that on the heterogeneous culture.

(2) There is evidence that the round type of colony is more sensitive than the crescentic, the 1/10 dilution of "Ec" killing off three-fourths of the bacilli of the round type in 2½ hours, whereas the bacilli of the crescentic type were not appreciably reduced in that time.

(3) Comparing the effect of the bacteriophage with that produced by the carbolic acid, it will be seen that, during the first 24 hours, the action of the bacteriophage is more powerful than a 1/500 dilution of carbolic acid.

EXPERIMENT III.—*To study the effect of the strongest bacteriophage on the autogenous culture during a longer period of time.*

This sample of bacteriophage was obtained after the ninth passage, and is referred to as "I."

Dilutions of this sample were made from 1/50 to 1/2,500,000 and put up with the autogenous culture at room temperature. Subcultures were made at the end of 22 and 47 hours. The tubes were then placed in the incubator for three hours to see if any resistant bacilli would grow up in the tubes which had so far proved sterile. Subcultures on agar were then made.

The results are shown in Table III.

TABLE III.—*To Show the Action of Bacteriophage "I" on the Autogenous Strain.*

Number of bacilli per 1 c.c., 115,250. Experiment performed at 20·5° C. for 46 hours, and finally at 37° C. for 3 hours.

Dilution of bacteriophage.	Subculture after 22 hours at room temperature.		Subculture after 46 hours at room temperature and 3 hours at 37° C.	
	Lactose.	Agar.	Lactose.	Agar.
1/50	— —	No growth	— —	No growth.
1/500	— —	"	— —	"
1/2500	— —	"	— —	"
1/5000	— +	1 "nibbled" colony.	+ +	1 "nibbled" colony.
1/25,000	— —	1 "	+ +	⊥
1/50,000	— +	⊥	+ +	+
1/250,000	+ +	+ +	...	+ + +
1/500,000	— +	⊥	+ +	2 "nibbled" colonies.
1/2,500,000	+ +	1 large colony	...	2 "

Note.—In lactose tube + = acid and gas. In agar tube + + + = normal growth, + + = 2/3 normal growth, + = 1/3 normal growth, ⊥ = 1/6 normal growth.

Summary of Table III.—(1) The dilutions 1/50, 1/500 and 1/2500 remained sterile. (2) It will be noted that from the appearances on agar the dilutions 1/2,500,000 and 1/500,000 appear to have a stronger action than the 1/250,000 dilution. This phenomenon had not been observed when dealing with the heterogeneous strain. The cause of this zonal effect will be discussed later, but it gives rise to a very real difficulty in comparing the strengths of samples of bacteriophage on the autogenous strain.

EXPERIMENTS DEALING WITH THE PRODUCTION OF LYSIN IN THE COLD.

It had been found in some experiments on the rate of action of bacteriophage that the sample "I," which has been used as the stock in all the following work, would act quite efficiently at 0°C., reducing the number of bacilli from 2935 per drop (0·02 c.c.) to 2 per drop in an hour's time. As no growth occurs in the control culture at this temperature it should be possible to find out whether the destruction of a large number of bacilli was in itself sufficient to increase the amount of lysis present.

EXPERIMENT I.—*To find out whether increase in the activity of a bacteriophage takes place when the sensitive organisms with which it has been in contact have been killed.*

Three flasks each containing 100 c.c. of broth were taken; one was kept as a control, and 2 c.c. of bacteriophage " I " added to each of the other two—a dilution of 1/50.

Two agar slopes of the autogenous culture which had been incubated for three hours were emulsified in 5 c.c. saline; 1·6 c.c. of this emulsion were added to each flask, rendering them distinctly cloudy.

One of the flasks containing lysin was put in the incubator, the other two were put in the cold room at 0°C.

At the end of two hours the incubated flask was distinctly less cloudy than the control in the cold room, while the experimental flask which had been in the cold room showed no clearing.

Next day the incubated flask had become very cloudy while the flasks in the cold room had not changed in appearance.

At the end of 21 and 41 hours 20 c.c. of each of the experimental flasks was filtered, and on the sixth day 20 c.c. from the incubated flask.

The activity of these filtrates was tested on the heterogeneous strain and the results are shown in Table IV. When comparing the action of the bacteriophage in these filtrates with that of the stock "I" from which they were derived, allowance must be made for the dilution of 1/50 which took place when 2 c.c. of the stock lysin was added to the 100 c.c. of broth.

TABLE IV.—*To Show the Action of the Filtrates Obtained in Experiments I and II.*

Tested on "Merrill" at room temperature, subcultures being made into lactose at end of 24 hours. Control bacteriophage "I" 1/100 – –; 1/1000 – –; 1/2000 – +.

Source of filtrate.	Final dilution compared with "I."	Day on which filtration took place.				
		1st.	2nd.	4th.		6th.
				Cold.	Incubated.	
Flask kept in incubator .	1/500 .	– –	– +	...	...	+ +
	1/5000 .	– –	+ +	...	...	+ +
	1/50,000 .	+ +	...	...	...	...
Flask kept in cold room .	1/500 .	+ +	+ +	+ +	– –	...
	1/2500 .	+ +	...	+ +	– –	...
	1/10,000 .	...	...	...	+ +	...

Summary of Table IV.—(*a*) Incubated flask: (1) It will be seen that there is a slight absolute increase in the bacteriophage-content at the end of 21 hours.

(2) At the end of 41 hours there was an absolute decrease.

(3) At the end of 6 days there was not enough lysin to be detected by this method.

(*b*) Flask kept in the cold room: (1) The bacteriophage disappeared rapidly, and at the end of 21 hours there was not enough to be detected by this method.

EXPERIMENT II.—*To find out the effect of incubation on a mixture of bacteriophage and bacilli which had been in contact at 0° C. for four days.*

On the fourth day, as no clearing had occurred in the lysin-containing flask in the cold room, the following experiment was made.

Two test-tubes of approximately equal size were selected and 20 c.c. of the contents of the experimental flask from the cold room put into each; a few drops were also placed on an agar slope.

One tube was put in the incubator while the other was returned to the cold room.

At the end of one hour there was distinct clearing in the incubated tube, and at the end of three hours it was practically clear; both samples were then filtered.

The agar slope made before incubation proved sterile, and it is of interest that although the organisms had been killed, no clearing of the culture occurred until incubation had taken place.

These filtrates were put up with strain "Merrill" and the results shown in Table IV.

Summary of the results obtained in Experiment II.—(1) The incubation of a mixture of bacteriophage and bacilli which had been kept in the cold and in which the strength of the lysin had become much reduced led to the reappearance of the bacteriophage in its original strength.

(2) No actual increase could be proved to occur, although a large number of bacilli had been killed and lysed.

EXPERIMENT III.—*To find out whether a large number of bacilli killed and lysed would give more evidence of increase in bacteriophage than a small number.*

It was thought possible that a sufficiently large number of bacilli had not been used in the previous experiment, which was therefore repeated in the following way:

An emulsion was made in 7 c.c. of saline of the growth from 7 agar slopes. Two flasks, each containing 100 c.c. of bacteriophage "I" diluted 1/50 in broth, were prepared; 0·5 c.c. of the bacillary emulsion was added to one flask, "W," and 5 c.c. to the other, "S." Both flasks were kept in the cold room for 10 days when samples from each were filtered, (1) when taken direct from the cold room, and (2) after three hours' incubation. Marked clearing took place in both the incubated samples but neither became entirely clear.

Plates made from the experimental flask which had received 5 c.c. of emulsion, and from a control flask to which no lysin had been added, showed that at least 200,000 bacilli per drop (0·02 c.c.) had been destroyed by the bacteriophage.

The filtrates were tested on the heterogeneous and on the autogenous strains and confirmed the results obtained in the previous experiments.

Summary of results of Experiment III: (a) *Filtrates made direct from the cold room.*—(1) The amount of bacteriophage in both flasks was reduced.

(2) The reduction was more marked in "S," the flask which had received the larger number of organisms.

(b) *Filtrates which had been incubated for three hours.*—(1) The activity of the bacteriophage had been restored to its original strength for the autogenous culture.

(2) With the heterogeneous culture the original activity was not entirely

regained, but there was an increase in activity as compared with the action of the filtrates made direct from the cold room. This was particularly the case with the incubated sample from flask " W " which had received the smaller number of organisms.

Experiment IV.—*To find out whether a higher temperature would favour the reappearance of the lytic substance.*

When flask " S " had been in the cold room for fourteen days two samples of 10 c.c. were taken and placed in two test-tubes of equal size. One tube was placed in a water-bath at 47° C. for two hours, while the other was kept in a bath at 58° C. for the same time. 5 c.c. of each sample was passed through a Berkefeld filter. These filtrates were then tested on strain " Merrill."

Result of Experiment IV.—The amount of lysin found in filtrates which had been heated to 47° C. and 58° C. for two hours was less than that found in filtrates which had been incubated for three hours.

Experiment V.—*To find out whether the filtration through a porcelain filter had removed the bacteriophage in the previous experiments.*

The remaining half of each sample of " S " which had been heated to 47° C. and 58° C. in Experiment IV was subcultured and found to be sterile. They were tested on " Merrill."

Result of Experiment V.—These samples did not show any greater activity than the filtered specimens from the same source.

Conclusions drawn from these experiments.—(1) Bacteriophage kept in the cold in contact with bacilli becomes inactive.

(2) The larger the number of bacilli present, the more completely is the bacteriophage action lost.

(3) The bacilli which have been treated in the cold with bacteriophage, even if some weeks old, undergo lysis subsequently on incubation, and the active substance reappears in the broth. This lysin is undiminished in strength for the autogenous bacillus.

(4) Temperatures higher than 46° C. do not favour the reappearance of lysin.

(5) There is no evidence that the death and subsequent lysis of a large number of bacilli can lead to an increase in the amount of bacteriophage present.

EXPERIMENTS TO INVESTIGATE THE DISAPPEARANCE OF BACTERIOPHAGE WHICH TAKES PLACE IN THE COLD WHEN IN CONTACT WITH SENSITIVE BACILLI.

Experiment I.—*To find out whether the bacilli have combined with the bacteriophage.*

Four centrifuge tubes were filled to a mark with broth from flask " S," now 15 days old, and a test-tube-full to act as control was kept at room temperature.

The flask was plated and found to contain 30,050 bacilli per drop (0·02 c.c.).

The tubes were centrifuged, the supernatant fluid pipetted off into a test-tube and the deposit washed three times with peptone-water. The washings were saved.

Two of the centrifuge tubes were now filled to the mark with sterile broth, the other two with the original supernatant fluid.

There were thus three preparations—

(1) The unaltered sample kept at room temperature denoted "R."
(2) The washed bacilli in fresh broth denoted "B."
(3) The washed bacilli in supernatant fluid denoted "S."

All three specimens were cloudy. They were placed in the incubator and at the end of three hours were almost clear.

After twenty hours' incubation all the tubes had become cloudy, and subcultures made from them on agar showed a mixture of large "nibbled" colonies and small transparent colonies.

The tubes were now filtered.

If it had been possible, by washing, to remove the bacteriophage which had been in contact with the bacilli, then, of these specimens, "B" ought to show no evidence of lytic action, "S" very little, viz. that due to the very small amount of bacteriophage left in the supernatant fluid which had not entered into combination with the organisms, and "R," the control, should show much the strongest bacteriophage effect.

The filtrates were tested on "Merrill" and the results are shown in Table V.

Table V.—*To Show the Lytic Action of the Filtrates obtained in Experiment I, tested on "Merrill" at Room Temperature: Subcultures made at the end of 24 hours.*

Control bacteriophage "I" 1/500 lethal, 1/2500 not lethal.

Source of sample.	Final dilution compared with "I."	Subcultures on lactose. "R" untreated.	"B" washed + broth.	"S" washed + supernatant.
Flask S, containing 1/50 "I" and 5 c.c. bacillary emulsion, cold room 15 days	1/500	— —	— —	— —
	1/2500	— —	— +	— —
	1/5000	+ +	+ +	— —

Summary of Table V.—(1) It was found that all three samples showed evidence of the presence of bacteriophage.

(2) Not only was a bacteriophage present, but all the filtrates showed a lysin which was more active than the control.

(3) This increase was most marked in the case of "S," which contained the bacilli which had been washed and put up with the supernatant fluid.

(4) The filtrate from the washed bacilli in fresh broth gave almost as strong a lysin as the control untreated organisms. It had not, therefore, been possible by washing to alter the relationship existing between the bacteriophage and the organisms.

(5) Why the washed bacilli which had been incubated with the original supernatant fluid should have produced the strongest lysin is not clear, but a possible explanation may lie in the number of bacilli which were present in

each specimen when they were placed in the incubator. Details regarding the plates which were made from these specimens are as follows:

Flask " S," which had been in the cold room for 15 days, contained 30,050 bacilli per drop (0·02 c.c.).

Time and treatment.	Number of bacilli per drop.		
	Sample " R."	Sample " B."	Sample " S."
Three hours at room temperature.	1000	45	1450
After 3 hours' incubation . .	2·5	1·6	2
,, 24 ,, ,, . . .	9,440,000	19,800,000	7,500,000

It will be seen that in each case a very large number of bacilli, presumably those least resistant, had been killed off at room temperature. A further decrease in the number present occurred during the first three hours of incubation. The subsequent development of the resistant type of bacillus led to a definite increase, as compared with the control, of the amount of lysin present.

Conclusions from Experiment I.—(1) There is evidence that, in the cold, bacteriophage enters into combination with sensitive bacilli.

(2) The lysin so combined cannot be entirely removed by repeated washing.

(3) An increase in the amount of lysin was associated with the proliferation of a very resistant type of bacillus.

EXPERIMENT II.—*To repeat the previous experiment at a lower temperature and without incubation of the bacilli.*

When flask " S " had been in the cold room for seven weeks a sample was centrifuged and washed three times with broth which had been cooled to 0° C., so that the temperature of the organisms might be kept as low as possible and lysis of the bacilli reduced to a minimum.

The deposit was sucked dry after each washing, so that some organisms were unavoidably carried over from the deposit. These fluids were kept.

After the third washing the deposit of bacilli was shaken up with broth. This, the original supernatant fluid, and the washings were cultured on agar. The tubes were then placed in the cold room.

All the cultures showed a growth of slightly " nibbled " colonies.

After 24 hours in the cold the tubes were heated at 58°C. for 1½ hours and found to be sterile.

These five preparations, the supernatant fluid, the three washings and the deposit of bacilli in broth, were tested on the autogenous culture.

Summary of the results obtained in Experiment II.—(1) Subcultures on agar from mixtures of these filtrates with the autogenous culture which had been kept in the cold room for 24 hours showed no evidence of bacteriophage.

(2) Subcultures on agar made after three hours' incubation of the mixtures all showed evidence of bacteriophage.

(3) After 28 hours' incubation of the mixtures this bacteriophage effect was increased, the most marked result being obtained in the tube containing the washed deposit of bacilli.

(4) The third washing, although it was a dilution of at least 1/6,000,000 of

the original fluid in the flask, showed much the same appearances as those given by the supernatant fluid.

Conclusions from Experiment II.—(1) This experiment confirms the previous one that in the cold bacteriophage combines with sensitive bacilli.

(2) Repeated washings do not entirely remove the lysin from the organism.

(3) It shows that the lysin carried over with the bacilli is able to produce a bacteriophage effect on a fresh inoculation of organisms.

(4) There was a very marked difference in the amount of lysin which could be demonstrated in this experiment and that found in the preparations in the first experiment, in which the resistant bacilli had been incubated, and had increased in number.

EXPERIMENTS DEALING WITH THE PRODUCTION OF LYSIN AT 37° C.

As all efforts to produce increase of bacteriophage without growth of the bacilli had failed, observations were made on the production of lysin which resulted when varying proportions of bacteriophage and bacilli were incubated together and samples filtered at intervals.

EXPERIMENT I.—*To investigate the production of lysin resulting from the action of varying strengths of bacteriophage on different numbers of organisms.*

Three flasks each containing 200 c.c. of broth were taken. To two of these, "A" and "B," 1 c.c. of bacteriophage "I" was added—a dilution of 1/100; to the third, "C," 1 c.c. of a 1/10 dilution, so that it contained 1/1000 lysin.

Two agar slopes of the autogenous culture which had been incubated for three hours were emulsified in 5 c.c. saline and 1·6 c.c. added to flasks "A" and "C," only 0·16 c.c. being added to "B."

The flasks were placed in the incubator and 20 c.c. of each filtered at the end of 3, 24 and 72 hours.

These filtrates were tested against "Merrill" (see Table VI).

TABLE VI.—*To Show the Action of the Filtrates obtained in Experiment I on the Heterogeneous Strain "Merrill," Tested at Room Temperature; Tubes Subcultured into Lactose at the end of 24 hours.*

The control bacteriophage "I" 1/500 – –, 1/2500 + +.

Source of filtrate.	Final dilution compared with "I."	Number of hours sample was incubated before filtration.		
		3 hours.	24 hours.	72 hours.
Flask "A" containing bacteriophage 1/100; bacillary emulsion 1·6 c.c.	1/500	– –	– –	– +
	1/5000	– –	– –	– +
	1/25,000	+ +	+ +	+ +
Flask "B" containing bacteriophage 1/100; bacillary emulsion 0·16 c.c.	1/500	– –	– –	– –
	1/5000	+ +	– –	– –
	1/25,000	...	+ +	+ +
Flask "C" containing bacteriophage 1/1000; bacillary emulsion 1·6 c.c.	1/5000	– –	+ +	...
	1/10,000	– –	...	...
	1/50,000	+ +	+ +	...

Summary of Table VI.—(1) An increase in the strength of the lysin was detectable in each case.

(2) When the amount of lysin added was 1/100, as in " A " and " B," the bacteriophage in the flask containing the larger number of bacilli " A " reached a maximum sooner and disappeared more rapidly.

(3) When the amount of lysin was 1/1000, as in flask, " C," and the number of bacilli the same as " A," the increase in lysin was distinct at the end of three hours, but at the end of 24 hours was hardly greater than the control.

A subculture on agar made from this flask at the end of three hours' incubation showed a few " nibbled " colonies, but no enumeration was made.

Doerr and Gruniger (1922), using a high dilution of lysin and a small number of organisms and estimating the strength of the lysin and the number of bacilli present in their mixture at frequent intervals, found that the bacilli proliferated normally for the first four hours and then rapidly decreased in number; the lysin remained unaltered for the first 2½ to 3 hours, and then rapidly increased in titre, reaching a maximum in 4 hours. The lysin increased 100,000-fold in two hours (titrated by Applemans' (1921) method).

I have calculated that I should have to use the bacteriophage " I " in a dilution of 1×10^{-7}, or 10^{-8} for it to approximate in value to the lysin used by Doerr and Gruniger. I hope to be able to carry out further experiments with such dilutions at a later date.

Conclusions from Experiment I.—Much more work requires to be done to determine the exact significance of these results, but they suggest that bacteriophage is reproduced either by a certain variety of the organisms or at a certain stage in their growth.

They show that favourable conditions may be produced by varying the amount of lysin, the number of bacilli, or the time at which filtration is carried out.

EXPERIMENT II.—*To see whether the resistant type of bacillus was suitable for the production of bacteriophage in a passage experiment.*

Subcultures were made from a culture of " nibbled " colonies which had been obtained from flask " A " at the end of 24 hours' growth, and an experiment exactly similar to that described undertaken, the flasks in this case being " AR," " BR " and " CR."

These filtrates were tested against both the heterogeneous and the autogenous strains.

Summary of results obtained from Experiment II.—(1) With the heterogeneous strain only the flask " CR " containing the 1/1000 lysin showed evidence of an increase in the amount of bacteriophage present.

(2) When tested on the autogenous strain all the filtrates showed increased activity of lysin.

(3) The effect of these lysins was observed over a period of 17 days. Several examples of late sterilisation of the culture occurred with high dilutions of lysin, confirming the previous observation that certain proportions of lysin and bacilli are more favourable for the production of bacteriophage than others. Ellis called attention to this zonal phenomenon as occurring when visible lysis

is taken as the criterion of bacteriophage effect. In my experiments it was particularly noticeable with lysins that were not strong enough to prevent the growth of the resistant type of bacillus, and especially when the tubes were kept at room temperature, so that changes in the number of organisms present took place slowly.

An example of the zonal effect is given in Table VII.

TABLE VII.—*To Show the Effect of the Filtrate Obtained from Flask "AB," Containing the Resistant Type of Bacillus and 1/100 Bacteriophage, Filtered after Three Hours' Incubation and Tested on the Autogenous Strain at Room Temperature.*

Final dilution compared with bacteriophage "I."	Subcultured on lactose after interval of —			Agar 17 days.
	1 day.	4 days.	17 days.	
1/5000	– –	– –	– –	No growth.
1/50,000	– +	– –	– –	"
1/250,000	– +	– –	+ +	Good growth.
1/500,000	– +	+ +	– +	No growth.

EXPERIMENT III.—*To find out whether the resistant type of bacillus without the addition of lysin could give rise to bacteriophage.*

A saline emulsion of some "nibbled" colonies obtained on subculture from flask "A" was made, and 0·7 c.c. put in a flask containing 100 c.c. of broth. No lysin was added. The flask was incubated.

Twenty c.c. of this culture was filtered at the end of 3 and 5 hours and on the second and fourth day.

These filtrates were put up against the autogenous culture (Table VIII). One series of tubes was kept at room temperature for 24 hours and then subcultured, the other series in the incubator for three hours.

TABLE VIII.—*To Show the Action of Filtrates Made from a Broth Culture of the Resistant Type of Bacillus on the Autogenous Strain.*

Length of incubation before being filtered and dilution.	Appearances of subcultures on agar.	
	Mixtures kept 13° C. 24 hours.	Incubator 3 hours.
3 hours 1/5	Very small "nibbled" colonies	12 "nibbled."
1/50	Good growth, many circles of "bacteriophage"	...
5 hours 1/5	A few small colonies	6 "nibbled."
1/50	Good growth, many circles	...
48 hours 1/5	A few very small colonies	No growth.
1/50	Good growth, many circles	...
96 hours 1/5	Good growth, many circles	3 small colonies.
1/50	Good growth, a few circles	...

Summary of Table VIII.—(1) All the subcultures on agar from both series showed evidence of the presence of bacteriophage.

(2) The sample which had been filtered on the second day was the most active.

Conclusions from Experiment III.—Without further experiments it is impossible to be certain whether this bacteriophage has been produced by the resistant bacilli, or has been carried over by them from the strong bacteriophage in flask "A" with which they have been in contact. Flask "A" was lethal in a dilution of 1/2500.

As there was evidence of an increasing amount of lysin up to the end of 48 hours, it is possible that bacteriophage was really produced by these organisms.

As, however, the resistant type of bacillus, on subculture, always tends to revert back to the non-resistant type, it is extremely difficult to devise an experiment to prove this point.

GENERAL CONCLUSIONS.

(1) Experiments to produce an increase in the strength of a given bacteriophage by allowing it to kill and subsequently lyse sensitive bacilli have proved negative.

(2) There is some evidence that increase in lysin is associated with proliferation of the resistant type of bacillus.

(3) There is evidence that the proportions existing between the strength of lysin used and the number of bacilli present, and the length of time that the lysins act, are all of importance in the production of bacteriophage.

(4) These observations explain the zonal phenomenon which is seen when varying dilutions of a bacteriophage are put up with the autogenous culture.

(5) Bacilli acted on in the cold by a bacteriophage enter into some combination with it. This has been shown to occur with dead organisms by Jaumain and Meulemain (1922). The bacteriophage is again set free on lysis of the bacilli and is increased in amount if the organisms are then allowed to proliferate.

(6) These observations explain the so-called "mixed" cultures which have been isolated from urine and their peculiar variations in growth.

This work was carried out during my tenure of a Beit Memorial Research Fellowship.

I have to thank Dr. Ledingham for having suggested this research, for having insisted on the employment of quantitative methods, and for much helpful advice and criticism throughout.

REFERENCES.

Appelmans, R.—(1921) "Le dosage du Bactériophage," *Compt. rend. Soc. de Biol.*, **85**, 1098.

Doerr, R., and Gruniger, W.—(1922) "Studien zum Bakteriophagen-problem I," *Zeitschr. f. Hyg.*, **97**, 209.

Ellis, A. W. M.—(1922) Discussion at the British Medical Association on Bacteriophage, *Brit. Med. Journ.*, **2**, 298.

d'Hérelle, F.—(1921) 'Le bactériophage, son *rôle* dans l'immunité,' Paris. (Masson et Cie.)

Jaumain, D., and Meuleman, M.—(1922) "Absorption du principe lytique par les microbes tués," *Compt. rend. Soc. de Biol.*, **87**, 362.

Lepper, E. H.—(1923) *Brit. J. Exper. Pathol.* (in the press).

Maitland, H. B.—(1922) "Experiments Employing a Quantitative Method in a Study of the d'Hérelle Phenomenon," *Brit. J. Exper. Pathol.*, **3**, 173.

Otto, R., Munter, H., and Winkler, W. F.—(1922) "Beiträge zum d'Hérelleschen Phänomen," *Zeitschr. f. Hyg.*, **96**, 118.

THE DETECTION OF *SPIRONEMA PALLIDUM.*

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Among the methods of detecting *Spironema pallidum* there is one that may be regarded as quantitative, and which, therefore, does offer reasonable certainty in diagnosis. This is that recommended by L. W. Harrison ('A Manual of Venereal Diseases,' p. 95), in which the relation of the number of spirals to a given length is determined. The standard suggested is the diameter of a red blood-cell, and seven complete coils of the spironema should be counted in this length. Any number more or less than this renders it improbable that *S. pallidum* is being observed, and when taken in conjunction with other known characteristics it may be regarded as definitely confirmatory. The difficulty of applying this method under ordinary circumstances is obvious, as either there may be few blood-cells in the material, or a long search may not result in seeing a cell and a spironeme in convenient proximity. With a view to overcoming this objection to an otherwise valuable aid the method to be described has been evolved, and has been found easy in manipulation and constant in results. The only appliance necessary, in addition to the ordinary microscopical equipment for dark-ground illumination, is a graticule, which is placed on the diaphragm in the ocular in the same way that an eye-piece micrometer is disposed. It is necessary to make sure that the position of the diaphragm is such that the pattern of the graticule is sharply defined when the ocular is in use. This is not invariably the case, and it may be necessary for some slight adjustment to be carried out by an optician to obtain the desired result. The pattern and size of the graticule is seen in Fig. 1. It consists of a photo-

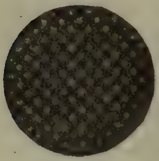

Fig. 1.

graphic image with a semitransparent ground on which a number of clear spaces are regularly disposed, so that a large proportion of these spaces are always in the field of view. The purpose is to so arrange matters that each clear circle occupies the same area in the field that would be occupied by a red blood-cell. It is obvious that this is dependent on the focal length of the objective in use, and to get a satisfactory result a graticule should be supplied of suitable pitch for each objective if more than one is generally used. The one figured is suitable for a Leitz $\frac{1}{12}$ in. oil-immersion suitably stopped down. In any case some slight adjustment is necessary before the method is ready for use. A normal red blood-cell should be observed, and if its image exactly fills one clear circle then adjustment is correct. Should it not do so the draw-tube of the microscope must be moved either up or down as required until the desired result is obtained. The necessary adjustment should not exceed five millimetres in either direction. Should more movement than this be required the graticule is not of the right pitch and should be exchanged by the makers for another. The correct tube-length with a particular objective and ocular having once been determined, it is only necessary to set it at the same position each time a search is to be made. When all is in correct adjustment the image appears as in Fig. 2. It will be seen that the spirals to be counted are brighter than those outside the circle and this greatly facilitates the counting. After some little experience it is possible to tell without actual counting whether the required seven coils are within the circle, or, what is equally important, whether more or less than this number are there. The method may be applied to other spirochætes where the spacing of the coils is characteristic. It is sometimes difficult with an indifferent mechanical stage to move the object exactly to the required position, but this can often be overcome by rotating the eyepiece when a circle is likely to cross the object.

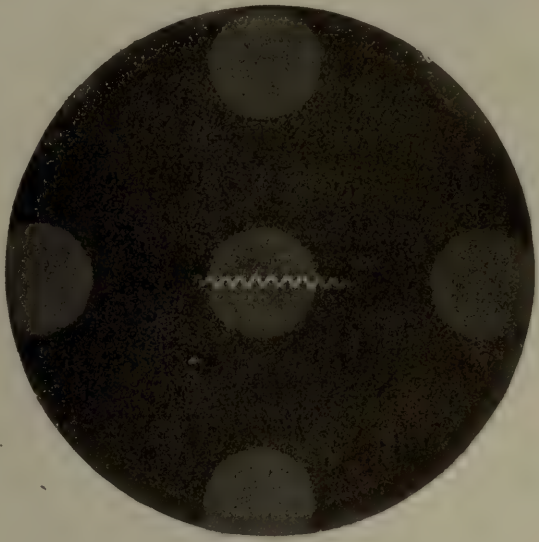

Fig. 2.

One possible source of error arises when a light of too great intensity is used. In this case irradiation from the blood-cell takes place, and it appears larger than it should do. In such a case the light must be reduced until the cell is only bright enough to be seen comfortably. This may be effected by interposing one or more pieces of neutral or coloured glass between the microscope and the illuminant.

Suitable graticules can be obtained from Messrs. Hawksley & Sons, Wigmore Street, W.

INVESTIGATION OF THE ÆTIOLOGY OF TYPHUS FEVER, ESPECIALLY UNDERTAKEN FOR THE EGYPTIAN GOVERNMENT IN THE PUBLIC HEALTH LABORATORIES, CAIRO.

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THE work of which the following paper is an account was carried out during the early months of 1922 in the Laboratories of the Public Health Department at Cairo. It was unfortunately never completed on account of the illness of both the workers from typhus fever. The observations and experiments made will be best considered under three headings: those made—

(1) On the blood of men and guinea-pigs infected with typhus fever.
(2) On the infection of lice with typhus virus and rickettsia.
(3) On lice collected from apparently healthy persons in Cairo.

The virus used for experimental purposes was obtained in the form of blood from two human cases of typhus fever.

OBSERVATIONS ON TYPHUS VIRUS IN BLOOD.

An attempt was made to determine whether the virus was especially closely associated with the blood-platelets. With this object the following experimental inoculations were made into guinea-pigs. All the inoculations of blood and platelets were made intraperitoneally. The sources of the typhus virus used and the experiments carried out were as follows:

Case 3.—M. S—, male, 24 years, admitted February 13th, which was supposed to be the 13th day of the disease, very ill with typhus fever; died February 14th. Weil-Felix reaction positive.

February 13th, 10 c.c. of citrated blood (9 c.c. of blood and 1 c.c. of 5 per cent. citrate of sodium) was received at the laboratory. 6 c.c. of the blood was centrifuged for 10 minutes, 3 c.c. of colourless turbid plasma was pipetted off and again centrifuged for 30 minutes till clear. The deposit examined microscopically in a thick drop after drying and staining consisted almost entirely of platelets; a few red blood-corpuscles and very few lymphocyte nuclei were seen. The deposit of platelets was emulsified in salt solution and made up to 3 c.c.

The following inoculations were made:

Guinea-pig 3 received on 13 . ii . 22, 3 c.c. of whole blood. Result negative, and a subsequent immunity test made on 5 . iv caused a typical febrile attack.

Guinea-pig 4 received on 13 . ii . 22, 2 c.c. of platelet emulsion. Result positive; on 24 . ii (11th day) a febrile reaction began and reached 40° C. Passage to Guinea-pigs 11 and 12 gave positive results and the virus was also carried on through 9 guinea-pigs in series.

Guinea-pig 5 received on 13 . ii . 22, 0·5 c.c. of platelet emulsion; result positive. On 24 . ii a febrile reaction occurred rising to 40° C. Passage was made with platelet emulsion from Guinea-pig 5 to Guinea-pig 9, which reacted well, but when tested for immunity gave a late reaction, causing some doubt as to the completeness of the immunity.

Guinea-pig 6 received on 13 . ii . 22, 0·1 c.c. of platelet emulsion. Result negative. No marked febrile reaction nor immunity resulted.

Case 4.—Admitted 1 . iii . 22 in the second week of illness; temperature 39° C. Weil-Felix reaction positive; recovery.

Experiments with blood and platelets of Case 4 on 2 . iii . 22 similar to those with the blood of Case 3 gave uncertain results, except that 1 c.c. of whole blood produced a good reaction in Guinea-pig 13, and from this animal the disease was successfully passed on in guinea-pigs in series to the seventh inoculation from man. The other inoculations, viz. 3·5 c.c. of whole blood and 0·1, 0·5 and 2·0 c.c. of platelet emulsion, were followed by results which were uncertain from various causes.

Guinea-pigs.—Inoculations were made with platelets from four typhus guinea-pigs: Guinea-pig 9 inoculated with 1·0 c.c. of platelets from Guinea-pig 5; result positive, immunity test doubtful. Guinea-pig 10 inoculated with 0·1 c.c. of platelets from Guinea-pig 5; result negative, no immunity. Guinea-pig 12 inoculated with 0·5 c.c. of platelets from Guinea-pig 4; result positive. Guinea-pig 32 inoculated with 2·5 c.c. of platelets from Guinea-pig 22; result positive.

The results with the blood of Case 3 tend to confirm the work of Kusama (1920) and Ségal (1922), which appeared to indicate that typhus virus in blood was found in a much greater concentration in a platelet emulsion obtained by centrifuging than in the other parts of the blood.

The leucocyte layer was not examined separately, since it is very difficult if not impossible to obtain the leucocytes in anything like purity.

The results with platelets from typhus guinea-pigs also confirm the observations of Ségal (1922).

Microscopic examination of whole blood, of leucocytes and platelets and of thick drops of platelet emulsion was carried out with material from the two human cases and from several of the guinea-pigs, but no fresh light was obtained on the question of the form taken by the virus in the blood. No reliable or constant variations from the normal could be established.

EXPERIMENTAL INFECTION OF LICE (*PEDICULUS HUMANUS*).

The lice used for these experiments were derived from a normal stock brought from England and fed on a healthy man. The lice after infection with typhus virus were fed on a typhus convalescent.

Feeding Normal Lice on Typhus Patients.

Lice in Box 5/2 were fed on Case 3 on 13 . ii . 22, which was stated to be the 13th day of the disease, and subsequently on a healthy person immune to typhus; result positive. Examination of the lice by dissection and smears showed *Rickettsia prowazeki* in one louse in the thread form on 23 . ii . 22 and in the minute form in one other louse on 2 . iii. Lice in Box 11/2 were fed on Case 4 for 11 days, beginning on 3 . iii, the day before the temperature fell; result negative. These lice never showed any definite infection with rickettsia, and 11 of the lice from this box inoculated on 15 . iii into Guinea-pig 34 caused a febrile reaction of a not very convincing character. The small amount of infection discovered in these two boxes was probably due to the short time of feeding on a febrile patient in each case, and perhaps the late stage in the illness of each patient was a contributory factor. The fact that lice were demonstrably infected with rickettsia on Case 3 but not on Case 4 corresponds to the readiness with which guinea-pigs were infected by inoculations of the blood of Case 3 and to the relative difficulty of infecting with the blood of Case 4.

Experiments on the Inoculation of Lice per anum *with Emulsions of Platelets.**

Experiment 1.—15 lice in Box 5/1 were injected on 13 . ii . 22 with an emulsion of platelets from Case 3. The emulsion was made by including the platelets obtained from 1 c.c. of turbid plasma in 10 drops of salt solution. Result, positive. On 11 . iii (26th day) one louse was found to be heavily infected with rickettsia.

Experiment 2.—28 lice in Box 5/3 were injected with a minute portion of the platelets from 3 c.c. of turbid plasma from Guinea-pig 5 emulsified in 6 drops of saline. Result positive.

On 3 . iii (6th day) one louse was found to be slightly infected and on 19 . iii one louse was heavily infected with rickettsia; in all four lice showed infection.

Experiment 3.—36 lice in Box 5/4 were injected on 27 . iii with a platelet emulsion from Guinea-pig 4. Result positive.

8 . iii (9th day): A few threads of *R. prowazeki* were found in excreta.

9 . iii: 1 louse showed many *R. prowazeki*.

18 . iii: 2 lice, and 19 . iii: 4 lice showed *R. prowazeki*.

Experiment 4.—9 lice in Box 5/6 were injected on 14 . iii with an emulsion of the platelets from 3 c.c. of plasma of Guinea-pig 22 suspended in 8 drops of salt solution. Result positive.

22 . iii (9th day): Excreta showed many *R. prowazeki*.

23 . iii: The excreta which had been passed in the last 24 hours and showed *R. prowazeki* were injected subcutaneously into Guinea-pig 47 with positive result, and a similar result also followed the injection of the excreta passed on 24 and 25 . iii.

Experiment 5.—26 lice in Box 11/1 were injected on 2 . iii with platelets from Case 4. Result negative. No rickettsia were found in any of these lice, and many of them were infected with bacteria. The last two lice died 18 . iii. This result is in conformity with the difficulty experienced in infecting guinea-pigs with the blood of Case 4, and also the failure to infect lice by direct feeding on this patient.

Lice in two boxes were directly infected with *R. prowazeki* by means of an emulsion made from previously infected lice. The severe infection which it was found possible to obtain by injecting them *per anum* with material of this kind enabled the thread forms of rickettsia and their intra-cellular position to be observed with great regularity.

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* The anal injection of lice was carried out exclusively by A. W. Bacot.

Experiment 6.—22 lice in Box 5/5 were injected on 9 . iii *per anum* with an emulsion of a louse from Box 5/4 which had been infected with platelets from Guinea-pig 4. Result positive.

12 . iii : 1 louse showed indications of an early infection.

14, 15 and 16 . iii : Lice were found definitely infected with *R. prowazeki.*

15 . iii : The excreta were injected into Guinea-pig 33, and caused infection which was further transmitted.

Experiment 7.—35 lice in Boxes 5/7 *a* and *b* were injected on 17 . iii with an emulsion of a louse containing many rickettsia from Box 5/5. Result positive.

22 . iii : Excreta showed rickettsia definitely from Box *b*.

30 . iii : Excreta from (*a*) show some granules, possibly rickettsia, and those from (*b*) show swarms of rickettsia. This difference in the contents of the two boxes was accounted for by the fact that Box 5/7*a* had been kept at about 20–22°C., Box 5/7*b* at 32–33°C.

Remarks.—Weigl (1920) and Bacot (1922) described the technique of injecting *Pediculus humanus per anum,* and Weigl (1920) recorded observations on the infection of lice with *R. prowazeki* by injecting them with the leucocyte layer of citrated blood from typhus patients.

Bacot and Ségal (1922) showed that lice could be infected by the same method, using as virus an emulsion of platelets from typhus guinea-pigs. The separated platelets from human cases of typhus, however, have not as far as can be ascertained been used before to infect lice *per anum.* The matter is of some interest in connection with the claims of Kusama and Ségal that the virus of typhus is contained in or is closely adherent to the platelets in the blood.

It is worth noting that it was found on trial that a *thick* emulsion, *e.g.* the platelets from about 3 c.c. of blood emulsified in six drops of saline, was required to produce a severe and certain infection, and that it was best to inject the emulsion alone and not to combine it with a feed of whipped blood, since sometimes clotting occurred in such a mixture.

The above method of determining the presence of the virus of typhus, *i.e.* by examining lice for rickettsia after they have been injected with platelets, appears to be one of the best ways of establishing that an attack of fever in a guinea-pig following inoculation is really due to typhus virus; for though the nature of rickettsia bodies has been critically examined by various writers, few who have worked at this aspect of the subject would deny the constant association of typhus virus and *R. prowazeki* in lice.

Examination of Lice which had been Injected with Virus.

(a) *Microscopic examination for rickettsia.*—It will be seen from the last sections that of five attempts to infect lice with *Rickettsia prowazeki* by means of platelets from blood presumably containing typhus virus, four were successful, viz. one experiment (Box 5/1) with platelets from a human case, and three (Boxes 5/3, 5/4 and 5/6) with platelets from guinea-pigs. One experiment (Box 11/1) was unsuccessful with human platelets.

Of the three batches of lice (5/5, 5/7, 5/8) injected with material from the guts of three previously infected lice all became infected with *R. prowazeki.*

Of two attempts to infect lice with rickettsia by feeding them on human typhus patients one was successful and the other failed, but in both cases the possible infecting feeds were very few (see above).

(b) *Inoculation of emulsions of lice into guinea-pigs.*—The lice which had been artificially infected were examined in certain instances for virus by injecting them or their excreta subcutaneously into guinea-pigs.

(1) Eleven dead lice from Box 11/2 in which no rickettsia bodies could be found after feeding on Case 4 were inoculated on 15 . iii into Guinea-pig 34. Result doubtful; a slight febrile reaction.

(2) One dead louse from Box 5/5 showing abundant rickettsia bodies was inoculated on 18 . iii into Guinea-pig 40, with a positive result, and the infection was carried on by passage to two other guinea-pigs.

Inoculation of Excreta of Infected Lice into Guinea-pigs.

Experiment 1.—15 . iii . 22: Excreta of 20 lice in Box 5/5 showing many rickettsia forms were injected into Guinea-pig 33 with a positive result; a further passage with the blood of Guinea-pig 33 to Guinea-pig 45 produced a short but definite attack of fever.

Experiment 2.—16 . iii . 22: Excreta of 18 lice in Box 5/5 showing very many rickettsia bodies were injected into Guinea-pig 38 with a positive result, and passage in series to two other guinea-pigs (53, 48) produced typical reactions in each case.

Experiment 3.—19 . iii . 22: Excreta of 10 lice in Box 5/5 showing very many rickettsiæ were injected into Guinea-pig 41 with a positive result, and a further passage to Guinea-pig 54 caused a slight reaction.

Experiment 4.—23 . iii . 22: Excreta of 17 lice in Box 5/6 showing many rickettsiæ were injected into Guinea-pig 47 with a positive result and further passages in series to Guinea-pigs 67, 81 and 92 were also successful.

The above four experiments were made with excreta which were collected from the box of lice on the same day that they were injected. The next experiment was made with excreta which had been kept in a dry condition for some days; it was unfortunately the only one accomplished of a series of such experiments which it was intended to carry out.

Experiment 5.—5 . iv . 22: Excreta of not more than 16 lice in Box 5/6 which were collected on 24 and 25 . iii and kept 11 to 12 days were injected subcutaneously into Guinea-pig 72 with a positive result. The excreta were examined microscopically and found to contain abundant rickettsia-bodies. The temperature of Guinea-pig 72 was raised from the 11th to the 20th day from the inoculation and was 41°–41·1° C. for 3 days. The blood of this animal inoculated into Guinea-pig 82 produced a positive result with a maximum febrile reaction of 41·4° C.

Discussion of the Results of Experimental Inoculation of Infected Lice and their Excreta into Guinea-pigs.

The only doubtful result from the injection of louse material followed the inoculation of an emulsion of 11 lice from Box 11/2 which had fed on Case 4.

The guinea-pig inoculated (No. 34) had a rise of temperature of only about half a degree centigrade with a maximum of 40° C. The emulsion used did not contain any demonstrable rickettsiæ. It is noteworthy that each of the five specimens of excreta which contained numerous rickettsiæ produced a febrile attack in a guinea-pig such as is characteristic of a typhus infection. Though Nicolle, Blanc and Conseil (1914) found that the excreta of typhus-infected lice were regularly able to reproduce the disease in guinea-pigs, this has not been the experience of all workers. Rocha-Lima (1916) declared that he could not obtain infection with louse excreta, and Atkin and Bacot (1922) failed to infect animals with excreta of lice fed on a typhus monkey.

The most interesting positive result in this series was that obtained by injection of Guinea-pig 72 with excreta which had been kept at least 11 days in a dry condition. These excreta were shaken as a dry powder through the fine gauze cover of the box containing the lice into a sterile Petri-dish and were kept in a cupboard in the laboratory, remaining apparently quite dry. The result is noteworthy, since it has been generally held that drying rapidly destroys the virus of typhus. The lice affording these excreta were no doubt infected to a very high degree on account of the material and the method used for infection. Most experiments to test the resistance of the virus to drying have, however, been made with blood, in which the virus appears to exist in a much lower state of concentration. In the experiments recorded by Nicolle and his colleagues (1914) the excreta which proved virulent were apparently collected daily and inoculated on the same day that they were collected, and Nicolle (1922) mentions that he considers that excreta become inactive after a very short time—certainly within 8 days.

CONTROL EXAMINATION OF LICE WHICH HAD BEEN FED ON APPARENTLY HEALTHY PERSONS.

(1) The stock of normal lice brought from England and fed on a normal person was examined from time to time and was always found free from rickettsia.

(2) Normal stock lice in Box 2/1 were fed regularly on a recovered typhus patient who also nourished the experimental typhus lice. This control box was kept at 30° to 35° C. between the feeds, and the lice were examined occasionally but never showed any rickettsia bodies.

(3) Lice collected from apparently healthy persons, labourers, etc., living in Cairo.

Lice from 12 such persons were examined. In three of these only a few lice (2, 6 and 9 respectively) were examined, but from the remaining nine at least 12 lice were examined.

The lice from eight persons were quite negative as regards rickettsia, from two definitely positive results were obtained, and in nine lice from two other persons some rather uncertain bodies were found out of 34 lice examined (3 out of 22 and 6 out 12 lice). It must be remembered that doubtful results in a search for rickettsia are of very little value, since the *débris* of cells, etc., in films readily simulate a few scattered individual bodies.

No results have been recorded here as positive unless very definite rickettsia forms were present in large numbers.

The two persons from whom lice showed definite rickettsiæ were a blind man, "L," and woman, "K," who were of the poorest class and lived together. Lice were collected from them and examined twice and three times respectively.

From the man 53 lice were examined on one occasion (February 22nd) and 13 were found positive; on the other occasion 17 days later (March 11th) 18 lice were taken and all proved negative. From the woman 17, 8 and 24 lice were taken on three different days (February 19th, 21st and 22nd), and 1, 6 and 5 lice were found positive on these three occasions respectively.

The characters (staining, morphology and arrangement) of the rickettsia bodies found in lice from these two persons were apparently identical. They differed, however, in certain particulars from the forms found in trench fever and typhus. (See Plate.)

The bodies were usually very small and in general appearance resembled *R. prowazeki*. The staining with Giemsa was as a rule pinker and less purple than in the case of *R. quintana*, and though many of the forms were extremely minute and round or diplococcal in shape, there was often a tendency for them to form rods containing deeply stained dots, or short wavy threads which were sometimes uniformly stained. Most of the round or oval forms were no larger than the smallest *R. quintana*.

The most notable feature in their arrangement was the tendency to lie in large or small clumps, as if agglutinated, and even when fairly well emulsified small clumps of 6 to 10 individuals usually remained and few were quite isolated.

It was found possible to make a fairly good emulsion in distilled water but clumps formed again very rapidly in 0·85 per cent. salt solution.

The films of dissected lice showed evidence of the rickettsia being included in cells of the stomach.

This kind of rickettsia is very like that described and figured by Weigl (1922) under the name of *R. rocha-limæ*. The arrangement in clumps is an especially noticeable point of agreement, as is also the intracellular position.

Other features which Weigl describes as distinguishing *R. rocha-limæ* from *R. prowazeki* are, a much greater diversity of shape, good staining properties with dilute aniline dyes, ready transmission naturally from one louse to another in the same box and even through the medium of boxes, etc., soiled with excreta, absence of virulence for guinea-pigs and a very low virulence for lice when injected *per anum*. The rickettsia which was found in Cairo agreed with Weigl's species in the absence of, or very feeble virulence for guinea-pigs, but not in the other particulars, except the clumped arrangement. The shape and size, though somewhat variable, did not cover nearly so wide a range as is often the case in *R. prowazeki*. Methylene blue or dilute fuchsin stained both species very feebly and about equally: normal lice in captivity did not become infected by contact, and *per anum* injection of both kinds into lice caused a rapid infection of the gut cells and early death of some individuals, but injection with the new variety appeared to be rather more fatal than with *R. prowazeki*.

The infection was passed on to six boxes of normal lice by injecting them *per anum* with emulsions of the gut of lice found naturally infected with the new kind of rickettsia.

Box 6/1: 24 lice injected from lice taken from "K"; large numbers of rickettsia were found in some of the lice on the 14th day from injection.

Box 6/2: 12 lice injected from lice in 6/1; positive on 7th day.

Box 7/1: Lice injected from a louse taken on "K"; positive on 7th day.

Box 7/2: Lice injected from a louse in Box 7/1; positive on 7th day.

Box 7/4: Lice injected from a louse in Box 7/2; positive 3rd day.

Box 7/6: 20 lice injected from a louse in Box 7/4; the excreta contained many rickettsia of characteristic appearance on the 4th and 6th days.

Source of Infection of the Lice with the Cairo Rickettsia.

No evidence was obtained that the infection passed naturally from louse to louse by copulation or by contact in the same box, but experiments in this direction were not completed.

Normal lice from Box 7/3 were immersed in a thick emulsion of rickettsia from lice of Box 7/2, but did not become infected.

Evidence of heredity in the louse was sought by examining the eggs of infected lice and first skin larvæ caught at the same time as infected adult lice.

The eggs on dissection showed no definite rickettsia, but several larvæ contained small groups of bodies believed to be rickettsia.

The attempt was made to determine whether the infected lice caught on "K" and "L" had derived the rickettsia from the blood of their hosts. Normal lice were accordingly fed regularly on these two persons.

(1) 22 . ii . 22: Uninfected lice in Box 6/3 were fed once daily on "K." 22 . iii . 22: No infected lice had been detected.

(2) 15 . iii . 22: 40 normal lice in Box 6/4 were fed on "K" daily but no infection could be found by 22 . iii . 22..

(3) 22 . ii . 22: Normal lice in Box 9/1 were fed daily on "L" and kept at 26°C. 28 . iii . 22: No rickettsia had been found so far.

(4) 27 . ii . 22: Some lice originally collected on "L" were put in Box 10/1 and daily fed on him, but up to 28 . iii . 22 no rickettsia could be detected in the excreta or dead lice.

It seems clear, therefore, that either the lice taken on "K" and "L" had not become infected by feeding on these persons and derived their rickettsia from some other source, or else that one or both of the hosts had recently harboured rickettsia in the blood but had recovered from the infection before the feeding experiments were begun.

The relation of the new species found in Cairo in lice from "K" and "L" to the previously described species in *Pediculus humanus* must remain at present uncertain. However, the possibility must not be overlooked that it may be a non-virulent variant of *R. prowazeki*, and the differentiating characters are not so definite as to exclude this possibility.

The experiments made to test the virulence of this form of rickettsia give very little encouragement to the hypothesis that a strain of *R. prowazeki* was present in these lice.

Though most of the inoculations were without effect, the reactions of two guinea-pigs—18 and 56—which occurred at the first passage from guinea-pig to guinea-pig were sufficient to encourage further work in this direction when another opportunity occurs.

CONCLUSIONS.

(1) The infectivity for guinea-pigs of an emulsion of platelets (nearly quite free from other blood cells) from a human case of typhus was high considering the small amount of blood material which it contained.

(2) The injection of lice *per anum* with an emulsion of platelets from a human case of typhus caused severe infection of the lice with *R. prowazeki.*

(3) The injection of lice *per anum* with emulsion of platelets from typhus guinea-pigs produced an infection of the lice with *R. prowazeki*, as previously shown by Bacot and Ségal.

(4) Lice infected with *R. prowazeki* were infective for guinea-pigs whether an emulsion of the lice themselves or of their excreta was used for the inoculation.

(5) The excreta of infective lice will remain active as a dry powder at laboratory temperature for 11 days.

(6) 204 lice from 12 apparently healthy persons were examined for rickettsia. 84 of these from 10 persons were negative (except for a few uncertain results).

(7) In films of 25 out of 120 lice taken from two apparently healthy persons who lived together rickettsia bodies were found.

(8) Lice from the two last-mentioned persons were only found to be infected during a period of four days, and lice taken from them subsequently were free, nor did fresh lice become infected by feeding on them.

(9) The rickettsia found in lice from these two persons resembled in some respects *R. rocha-limæ* and *R. prowazeki.* One of the most obvious special features was the occurrence of the minute forms in large dense clumps—an unusual arrangement in the case of *R. prowazeki* and *R. quintana,* but characteristic of *R. rocha-limæ.*

NOTE ON THE CAUSE OF LABORATORY INFECTIONS WITH TYPHUS.

The exact manner in which laboratory workers have become infected with typhus fever has not always been clear. It has usually been accepted that an actual transference of at least one infected louse from the sick to the healthy has occurred. In many cases no doubt a risk of acquiring infected lice has been incurred by contact with patients.

In the present instance no such opportunity occurred up to the time when the first laboratory infection (A. W. B., onset March 26th) was acquired, since neither of the two workers had recently seen a case of typhus nor been to the fever hospital. They had merely received infective material at the laboratory and had propagated the virus in guinea-pigs and lice. The second person attacked (J. A. A., onset April 5th) had visited A. W. B., but only in a perfectly clean hospital ward. There is no reason to suppose that any infected lice had escaped from boxes in the laboratory; indeed, the reverse is practically certain.

It has been widely held that the disease is usually communicated by the actual bites of lice, but very few recorded observations have been made to test this hypothesis in such a way as to exclude infection by means of the excreta of the lice. Atkin and Bacot (1922) made two careful attempts to infect macacus monkeys by feeding infected *Pediculus humanus* on them, and one similar attempt to infect a guinea-pig, taking care that the infection should not take place by inoculation of the excreta. None of these three animals became infected. There is no microscopic evidence suggesting that rickettsia might be transferred from the louse by the act of biting, nor can these bodies be demonstrated in the fore-part of the gut, mouth parts, or salivary glands.

The infectivity of excreta had become impressed on the workers in the present investigation by their proved virulence for guinea-pigs which were inoculated on March 15th and 16th, and a special investigation as to the dose and keeping properties of infective louse excreta had been planned and begun when A. W. B. became ill.

That the virus in louse excreta can readily enter the body through abrasions or slight wounds is very probable, and this has been shown to happen in the analogous case of trench fever. An unsuccessful attempt to infect a guinea-pig with the excreta of lice fed on a typhus monkey has been recorded by Atkin and Bacot (1922), but it is most probable that the infection is much more readily produced in man than in animals.

Töpfer (1916), in speaking of the death of Schüssler following a laboratory infection with typhus, says that in all probability the virus was introduced into the eye or mouth by the finger. He states, moreover, that Schüssler had an inflammatory affection of the lids, which indicated a possible place for the accidental inoculation to take place.

On the whole, then, it seems probable that excreta of infected lice are one, if not the chief source of laboratory infections with typhus, but of course other means of infection cannot be ignored, such as a prick with an infected dissecting needle.

Thanks are due to Dr. Charles Todd, the Director, and all the staff of the Public Health Laboratories, Cairo, for their courtesy and help; and also to Dr. Sami Sabongi, Medical Officer in Charge of the Fever Hospital, Abbassia, for his assistance in providing us with infective material, and help in other ways.

REFERENCES.

Atkin, E. E., and Bacot, A.—(1922) "Experiments on the Infectivity of Typhus Virus," *Brit. J. Exper. Pathol.*, **3**, 196.

Bacot, A. W.—(1922) "Details of the Technique Adopted in following Weigl's Plan of Feeding Lice Infected with the Virus of Typhus Fever by Rectal Injections," *Brit. J. Exper. Pathol.*, **3**, 72.

Bacot, A., and Ségal, J.—(1922) "The Infection of Lice (*Pediculus humanus*) with *Rickettsia prowazeki* by the Injection *per rectum* with the Blood-platelets of Typhus-infected Guinea-pigs and the Re-infection of other Guinea-pigs from these Lice," *Brit. J. Exper. Pathol.*, **3**, 125.

Kusama, S.—(1920) "On the Nature of the Virus of Typhus Fever," *Japan Med. World*, **10**, 125.

NICOLLE, C.—(1921) "L'infection par le virus du typhus exanthématique est-elle héréditaire chez le pou?" *Arch. de l'Inst. Pasteur de l'Afrique du Nord*, **1**, 433.

NICOLLE, C., BLANC, G., AND CONSEIL, E.—(1914) "Nouvelles recherches sur le typhus exanthématique," *Arch. de l'Inst. Pasteur de Tunis*, **9**, 85.

ROCHA-LIMA, H.—(1916) "Zur Ætiologie des Fleckfiebers," *Berlin. klin. Wchnschr.*, **53**, 567.

SÉGAL, J.—(1922) "The Association of the Virus of Typhus Fever with the Various Blood Elements," *Brit. J. Exper. Pathol.*, **3**, 95.

TÖPFER, H.—(1916) "Der Fleckfieber-erreger in der Laus," *Deutsch. med. Wchnschr.*, **42**, 1251.

WEIGL, R.—(1920) "Studies on *Rickettsia prowazeki*," *Przeglad Epidemjologiczny*, **1**, 15.—(1921) "Further Studies on *Rickettsia rocha-limæ* (n.s.)," *Przeglad Epidemjologiczny*, **1**, 375. (See also ROSENBERGER, G.—(1922) "Studien über die in- und extrazellularliegenden Rickettsien," *Arch. f. Schiffs- und Tropenkrankh.*, **26**, 112.)

DESCRIPTION OF PLATE.

Drawings made from stained films of emulsions from the guts of lice infected with *Rickettsia sp.?* found on two apparently healthy persons in Cairo.

FIG. 1.—From gut of dead louse from Box 7/1 injected with emulsion of the gut of another infected louse on 23 . ii . 22; film made 6 . iii . 22—11th day.

FIG. 2.—From gut of louse of Box 7/2 injected 4 . iii . 22 with emulsion of a louse of Box 7/1; film made 13 . iii . 22—9th day.

FIG. 3.—Film from gut of louse of Box 7/2; killed, and film made 7 . iii . 22—4th day.

FIG. 4.—Low-power view of the same film from which fig. 3 was taken, showing characteristic arrangement in clumps.

FIGS. 1–3 approximately × 1000.

THE BRAIN AND IMMUNITY REACTIONS TO JENSEN'S RAT SARCOMA.

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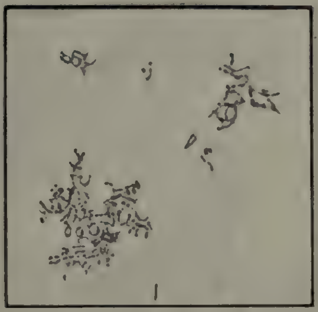
IN the course of experiments with Jensen's rat sarcoma, which consisted in simultaneous inoculation of grafts into various tissues of the body, a difference between rats in which the brain was inoculated and those in which it was not inoculated seemed to manifest itself. Thus of 25 animals in which brain inoculation was omitted, only 5 (20 per cent.) showed absence of measurable growth at the site of subcutaneous inoculation, whereas 11 animals in which brain inoculation was performed showed absence of measurable growth at the site of subcutaneous inoculation in 5 (45 per cent.), and a similar absence of growth in other organs into which growths were made. Though grouped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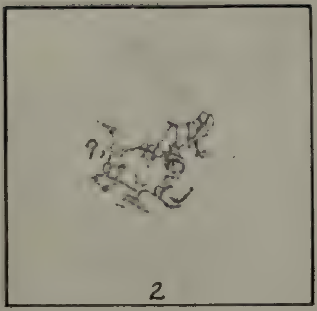
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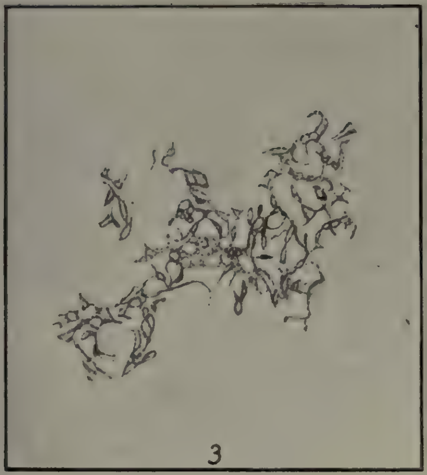
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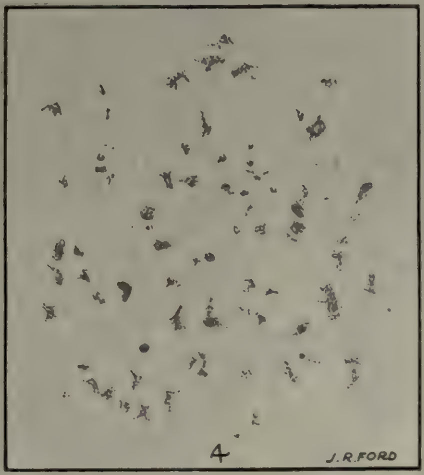
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Arkwright and Bacot.

together for brevity these results were not obtained on a single occasion, and though they might be referable to injurious effects of the cerebral operation upon general nutrition, it seemed desirable to test the matter under stringent conditions owing to its possible bearing upon the production of an active immunity in malignant disease.

A tumour was taken, which by synchronous experiment was seen to grow rapidly and take subcutaneously in 100 per cent. of cases, and the following experiment was made:

A. In 20 normal rats brain puncture without introduction of graft was performed.

B. In 20 normal rats grafts were introduced into the brain.

C. In 20 normal rats grafts were introduced into the right flank.

Seven days later the 15 survivors of A, the 16 survivors of B and the 20 survivors of C were inoculated into the left flank with a tumour which by synchronous experiment was seen to grow rapidly and take subcutaneously in 95 per cent. of cases. On the 10th day after this second inoculation the left sides of all the animals were examined for growth, and it was found that in A 12 (80 per cent.), in B 9 (56 per cent.), in C 15 (75 per cent.) showed measurable subcutaneous tumours. Taking the volume of a tumour as being represented by the value (length × breadth) $\frac{3}{2}$, the mean amount of tumour per rat of series A was 290 c.mm.; of series B, was 141 c.mm.; of series C, was 148 c.mm.; and of the controls, was 228 c.mm.

At the end of 5 weeks from the first inoculation, 3 rats in series A and 3 in series B were living, healthy and tumour-free, whereas all of the animals in series C were dead as the result of tumour growth.

The Jensen tumour selected took so readily and its growth was so rapid even in the brain that a hypothetical immunity mechanism was placed under the most adverse circumstances, for it needs but a small tumour (*e.g.* 5 mm. in diameter) within the skull cavity to kill the animal by pressure. Hence it was determined artificially to lower the vitality of the tumour used for inoculation in the hope of allowing more time for the brain to respond to the inoculation or to accommodate itself to a growing graft. With this object a tumour which in synchronous experiment on the 10th day had taken subcutaneously in 92 per cent. of cases and yielded on an average 476 c.mm. of growth per rat of the series, was exposed to X-rays under standard conditions for 25 minutes.* On the tenth day after subcutaneous inoculation grafts of this irradiated material had taken in 85 per cent. of cases and yielded on an average 244 c.mm. per rat of the series. The original experiment was repeated under the new conditions. Twenty normal rats (series D) received intracerebral grafts of the irradiated tumour, and 20 normal rats (series E) received irradiated tumour in the right flank. Seven days later the 17 survivors of D and the 20 survivors of E received into the left flank grafts of a subcutaneous tumour which during its whole existence had grown rapidly and at exponential rate, and by synchronous experiment was shown to take subcutaneously in 80 per cent. of cases and yield on the tenth day a mean value of 262 c.mm. per rat of the series. On the tenth day after the second (subcutaneous) inoculation it was found that of the primarily brain-inoculated animals (D), 13 (76 per cent.) showed measurable subcutaneous tumours with a mean volume of 351 c.mm. per rat of the series, while the primarily flank-inoculated animals (E) showed 70 per cent. of measurable secondary subcutaneous tumours with a mean volume of 346 c.mm. for each rat of the series. As said above, the control value was 262 c.mm. At the end of 5 weeks from the first inoculation all the animals of series D and E were dead, while 20 per cent. of the controls were living and without tumour.

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* For all the irradiations carried out in this investigation we are indebted to Miss Gladwys Scott. The "standard conditions" were: Distance of the tumour from the anticathode of a Coolidge tube 30 cm., alternative spark-gap 5 cm. between spheres 5 cm. in diameter, output of bulb checked every minute by electroscopic readings. Increased impairment in vitality of graft by irradiation was aimed at by giving 25 minutes, 35 minutes and 55 minutes' exposure respectively.

Examination of the results in series D and E, therefore, shows that both primary brain-inoculation and primary flank-inoculation with tumour irradiated under standard conditions for 25 minutes actually led to a greater volume of tumour, on test-inoculation, per rat of the series than in the controls, while any reduction in percentages of takes was negligible. Consequently it appears that primary inoculation of tumour irradiated with this intensity has favoured subsequent growth in the subcutaneous tissue instead of impairing it as was anticipated.

Since it appeared from series D and E that a local delay of growth (*cf.* below) might be associated with a general increase of sensibility, it was determined to increase further the degree of irradiation. Hence series F and G with tumour irradiated for 35 minutes under standard conditions, and series H and I with a tumour irradiated for 55 minutes under standard conditions, were carried out on identical lines with those already described. The test (subcutaneous) inoculation was made with a tumour which synchronous experiment showed to produce measurable tumours in 85 per cent. of cases, and to yield on the tenth day a mean value of 766 c.mm. per rat of the series. The results on the tenth day after test inoculation were as follows: In F measurable subcutaneous tumours were found in 17 out of 20 survivors (85 per cent.), and the mean volume of tumour per rat of the series was 590 c.mm.; in G, 13 out of 19 survivors (68 per cent.) showed measurable tumours and the mean volume of growth was 331 c.mm. per rat of the series; in H, 15 out of 21 survivors (71 per cent.) showed measurable tumours and the mean volume of growth was 476 c.mm. per rat of the series; while in I, 9 out of 19 survivors (47 per cent.) showed measurable tumours and the mean volume of growth per rat of the series was 178 c.mm. At the end of 5 weeks from the first inoculation, in series F, 1 rat was living, healthy and tumour-free; in series G there were 2; in series H there were 6; and in series I there was 1.

BEARING OF EXPERIMENTS UPON THE QUESTION OF *GENERAL* RESISTANCE.

For purposes of comparison, the mean amounts of tumour per rat of the various series may be expressed as percentages of the corresponding mean amounts of the controls. If this be done the values set forth in Table I are obtained.

TABLE I.—*Mean Volumes of Tumour on the Tenth Day in Test (Subcutaneous) Inoculations after a Preliminary Treatment of the Animal by Intra-cerebral or Subcutaneous Inoculation.*

(The values are expressed as percentages per rat of series of the corresponding mean volumes in the controls.)

Site of preliminary inoculation.	Treatment of tumour used for preliminary inoculation *Nil.*	 25 minutes' irradiation.	 35 minutes' irradiation.	 55 minutes' irradiation.
Brain	62	134	77	62
Subcutaneous (flank)	65	132	43	23

From these values it appears (1) that a previous inoculation with non-radiated tumour—whether intra-cerebral or subcutaneous—leads to a certain amount of general resistance to growth of a subsequent inoculation; (2) that a previous inoculation with tumour that has received a 25 minutes' dose of X-rays—whether intra-cerebral or subcutaneous—leads to a great diminution of general resistance to growth of a subsequent inoculation; (3) that previous inoculation with tumour that has received a 35 minutes' or a 55 minutes' dose of X-rays—whether intra-cerebral or subcutaneous—leads to a diminishing susceptibility to growth of a subsequent inoculation, which in the case of preliminary subcutaneous inoculation passes into the acquisition of a definite amount of increased general resistance or immunity. The difference in degree between the effect of previous intra-cerebral and that of previous subcutaneous inoculation with the 35 and the 55 minutes' irradiation of the graft we ascribe to the injurious effects of cerebral puncture, for an examination of Series A will show that cerebral puncture alone induces a certain amount of diminished general resistance to growth of a subsequent inoculation, and therefore counteracts to some extent the immunity conferred by irradiated tumour.

Examination of the numbers of healthy and tumour-free animals in the various series at the conclusion of the experiments (5 weeks after the initial inoculations) bears directly upon the question under discussion. They are given in Table II. The number of animals theoretically expected to be living at the end of the experiment was determined from the percentages of "takes" in the control animals for the different samples of tumour used as grafts. Thus, if 100 rats were inoculated with an 80 per cent. tumour and a week later with a 90 per cent. tumour, 80 rats would ultimately succumb to the first inoculation, and $\frac{90}{100}$ of the remaining 20 rats (*i.e.* 18) would ultimately succumb to the second inoculation. Hence at the end of the experiments, theoretically only 2 animals would be living.

From Table II it appears that a greater degree of general immunity is conferred upon the animal by a preliminary intra-cerebral inoculation with non-radiated tumour or tumour irradiated under standard conditions for 55 minutes than by a preliminary subcutaneous inoculation with similar material. The effect produced by intra-cerebral inoculation upon general resistance is further indicated by the behaviour of the test inoculations in the 10 rats that survived. In 4, the graft failed from the first; in 6, a tumour, often of a considerable size (*e.g.* 12 × 15 c.mm.), was formed, but subsequently underwent complete retrogression or degeneration.

TABLE II.

Series.	Site of preliminary inoculation.	Treatment of graft in preliminary inoculation.	Treatment of graft in test inoculation (subcutaneous).	Living, healthy and tumour-free animals at end of experiment.	
				Theoretical.	Actual.
B	Brain	*Nil*	*Nil*	None	19 %
C	Flank	,,	,,	,,	None
D	Brain	25 minutes' irradiation	,,	,,	,,
E	Flank	,, ,, ,,	,,	,,	,,
F	Brain	35 ,, ,,	,,	4 %	5 %
G	Flank	,, ,, ,,	,,	,,	11 %
H	Brain	55 ,, ,,	,,	9 %	29 %
I	Flank	,, ,, ,,	,,	,,	5 %

BEARING OF THE EXPERIMENTS UPON THE QUESTION OF LOCAL RESISTANCE.

The ultimate percentage of "takes" shows no difference according as the graft is intra-cerebral or subcutaneous. But if the amount of growth as the result of primary inoculation be considered it is seen that locally a great difference manifests itself. Thus, in spite of the fact that all primarily brain-inoculated animals in Series D (25 minutes' irradiation) ultimately died from cerebral tumour, yet on the twentieth day after inoculation 50 per cent. of them were living and showed no evidence of a brain lesion, whereas all the primarily flanked inoculated animals (Series E) showed a tumour at the site of inoculation so large that if one-tenth had been present within the brain the animals would have been moribund.

So also on the twentieth day after inoculation 75 per cent. of the primarily brain-inoculated animals in Series F (35 minutes' irradiation) and 100 per cent. in Series H (55 minutes' irradiation) were living and showed *no* evidence of cerebral lesion, whereas 77 per cent. and 43 per cent. of the primarily flank-inoculated animals in the corresponding Series G and I showed considerable masses of growth.

Similarly with non-irradiated grafts (Series B and C), 50 per cent. of the primarily brain-inoculated animals were living and showed no sign of a brain lesion on the twentieth day after primary inoculation, whereas all the primarily flank-inoculated animals showed a large local tumour. Hence it is clear that whether irradiated or not the early stages of an intra-cerebral graft are distinctly impeded as compared with the early stages of a graft made into the subcutaneous tissue. Delay of a graft to grow may depend upon (*a*) absence of suitable growth conditions or (*b*) presence of an antagonising mechanism, and on this question the experiments quoted above give no information. But a certain amount of information is afforded by histological examination of the cerebral grafts and tumours.

HISTOLOGICAL RESULTS.

Microscopical examination of the brain was made in 130 cases, of which 19 are excluded because the animals died shortly after operation. The remainder consisted of brain puncture without inoculation of graft, 14 cases; intra-cerebral inoculation of non-radiated graft, 25 cases; of graft irradiated for 25 minutes, 18 cases; of graft irradiated for 35 minutes, 20 cases; of graft irradiated for 55 minutes, 17 cases; intra-cerebral inoculation of non-irradiated grafts into immune animals, 11 cases; and normal brains of immune rats, 6 cases.

The results of inoculation at death of the animal are shown in Table III:

TABLE III.

Graft.	Active growth present.	Degenerated growth present.	Track alone present.	Total.
Non-irradiated.	18	6	1	25
25 minutes X-rays.	17	0	1	18
35 ,, ,,	18	0	2	20
55 ,, ,,	9	6	2	17

In those cases in which active growth was present, the histological characters of the cells, the number of mitoses, the vascularity, the absence of degeneration resembled these features in actively growing subcutaneous grafts, but the growing edge offered a considerable difference. In subcutaneous tissue the tumour resulting from a graft of Jensen's rat sarcoma shows few finger-like processes at the periphery or none at all; in the brain, on the contrary, such a tumour shows extensive finger-like invasion of the adjacent tissue except where it reaches the meninges or lateral ventricle. A very few lymphocytes or small mononuclear cells may lie in advance of the growing edge, especially where it reaches the meninges, but where brain substance is being invaded there is a remarkable absence of adventitious cells.

It follows from this description, which agrees in all essential points with the findings of Da Fano (1912), that the developing tumour in brain offers no evidence that the local nutritive conditions for growth are wanting.

In degenerating or fully degenerated grafts, or in tracks whence the graft or tumour has disappeared, a different picture is presented. Cells with special features are almost invariably found—often in great numbers. These cells have a diameter of 12 microns or more, are often well-defined, possess an excentric, round or oval, pale, diffusely staining, nucleolated nucleus, and a sparsely granular cytoplasm that stains faintly with pyronin. The nuclei differ little from one another but the cytoplasm appears to undergo disintegration readily, so that the outlines of the cells may be ill-defined and naked nuclei may lie in a granular *débris*. The cells may be discrete or may be aggregated into a veritable tissue which somewhat resembles the cortical substance of adrenal. Such an accumulation of cells surrounds a focus of degeneration more or less completely and separates it from the brain substance. A few lymphocytes or small mononuclear cells may be interspersed amongst the large special cells under consideration, but polynuclear leucocytes are absent. In the form which has been described above the finely granular cytoplasm does not contain any ingested material, but other cases occur in which the cytoplasm of large—apparently identical—cells is crammed with *débris* of red blood-corpuscles. This occurs notably in the track of a puncture without inoculation or a track after disappearance of the graft. Where the amount of blood *débris* is small the nucleus of the cell is distinguishable, but usually it cannot be detected (Fig. 1).

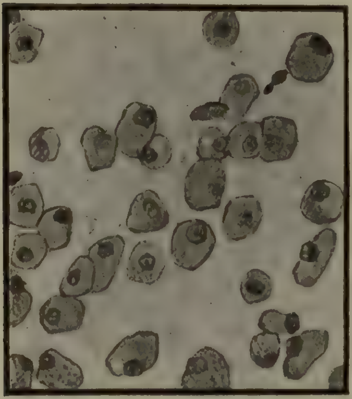

Fig. 1. × 600.

We are not in a position to affirm the nature of these cells. In many respects they bear resemblances to the so-called "macrophages" and may be associated with typical plasma-cells. They give no lipoid reaction, they are never found within blood-vessels, they are not altered tumour-cells, since they occur in the track of a brain puncture without inoculation of graft,* they are not found earlier than the fifth day after inoculation, they are usually associated with degeneration of the graft or with healing, but in two cases we have found them in advance of the growing edge of a graft of lowered vitality (35 and 55 minutes' irradiation respectively). The fact that we have found them in advance of a growing edge suggests to us that they are not merely a sequel to degeneration, but form part of an active reaction to tumour growth whereby degeneration of that growth is brought about.

CONCLUSIONS.

In the case of Jensen's rat sarcoma and the normal rat:

(1) Intra-cerebral inoculation of non-radiated or heavily-radiated grafts induces a subsequent increased general resistance to inoculation which is greater than that induced by inoculation of similar material subcutaneously.

(2) Intra-cerebral or subcutaneous inoculation of less heavily irradiated grafts induces a subsequent increased general sensibility to inoculation.

(3) The growth of a graft within the brain is restrained and delayed as compared with that of one in the subcutaneous tissue. This restraint and delay results in a greater frequency of degeneration and disappearance of the intra-cerebral graft, perhaps after a preliminary period of growth.

(4) The degeneration of an intra-cerebral graft or growth in the normal (and in the immune) rat is associated with the presence of large special cells which are derived from the host, and apparently form part of the process whereby that degeneration is brought about.

REFERENCES.

Da Fano, C.—(1912) *Fol. Neurobiol.*, **6**, 109.
Macklin, C. C., and Macklin, M. T.—(1920) *Arch. Neurol. and Psychiatry*, **3**, 353.

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* Cells, apparently identical, were found by the Macklins (1920) in repair of brain after aseptic lesions produced by hot or cold needles.

THE RELATIVE CONCENTRATION RATIOS OF SOME CONSTITUENTS OF THE URINE.

I.—THE DISTRIBUTION BETWEEN PLASMA AND CORPUSCLES, AND THE CONDITION IN THE BLOOD, OF UREA, CREATININE, INORGANIC PHOSPHATE AND URIC ACID.

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DURING the last few years observations have been accumulating, which suggest that the "filtration reabsorption" theory of renal secretion, put forward with a large body of evidence in its favour by Cushny (1917), in his monograph, requires modification. Investigations have been along two main lines: first, a direct examination of the passage of substances through the kidney of the frog; secondly, a larger number of observations of a more indirect nature on both man and animals.

Accepting the division of the constituents of the urine into the two groups of "threshold" and "no-threshold" bodies, it is clear that if two of the latter group simultaneously present in the blood are filtered off through the glomeruli and subsequently undergo concentration in the tubules from the absorption of water, the degrees to which they are concentrated should be the same in each case. This "concentration ratio" can be best expressed as the ratio of the quantity per cent. in the urine to the quantity per cent. in the plasma (MacLean, 1921). It is only the amount in the *plasma* which is available for filtration through the glomeruli, and then only if the substance in question is present in a "free," *i.e.* in a dialysable condition. This involves a consideration of the condition and distribution in the blood of the substances used in this work. Urea, inorganic phosphate, creatinine and uric acid were chosen.

The first three are described as true "no-threshold" bodies, while uric acid occupies a somewhat intermediate position between the no-threshold and threshold groups (Cushny, 1917). The selection of these substances was partly governed also by the availability of methods of analysis applicable to small quantities of blood and urine. Furthermore, except in the case of uric acid (Jones, 1920), these bodies undergo no further change in the animal organism. Marshall and Davis (1914) have shown this experimentally for urea; Cathcart (1916) and Barnett and Addis (1917) found, however, an increase in the ammonia of the tissues after the injection of urea.

A recent paper by Wu (1922) on the distribution of various substances between plasma and corpuscles in man gives a review of the previous literature.

He obtained an even distribution of urea, thus confirming earlier investigators, but found less creatinine in plasma than in corpuscles. The previous work of Wilson and Plass (1917), and Hunter and Campbell (1918), however, had shown an equal distribution for this substance also: and Wu states that the method used (precipitation method of Folin and Wu, 1919) may give too high results when applied directly to corpuscles. The former investigators estimated creatinine in whole blood and plasma and so obtained the amount in the corpuscles by calculation; they also allowed for the errors in the picric acid precipitation method, used on whole blood; Wilson and Plass, moreover, drew their conclusions from estimations performed after precipitation of proteins by heat coagulation.

As regards inorganic phosphate, Bloor (1918) found that there was twice as much in the corpuscles as in the plasma, but Zucker and Gutman (1922) observed a uniform distribution. The ease with which organic phosphorus is hydrolysed in acid solutions, such as are frequently used for protein precipitation, renders the determination difficult.

As regards the uric acid, which was used in a few experiments only, Wu (1922) found a higher value for the plasma than for the corpuscles; Theis and Benedict (1921) found an equal distribution in half of the cases examined, and in the majority of the remainder a higher amount in the plasma; Morris and MacLeod (1922) obtained about twice as much in the serum as in the corpuscles.

Turning to the question of the condition of these substances in the blood, Cushny (1919) has shown that urea and phosphate are completely dialysable, and should, therefore, be filterable through the glomerular membrane. Creatinine is also assumed to be present in the "free" state. As regards uric acid, it is suggested that a combined form exists in blood as well as the free acid, or free urate, but opinions differ as to how far the combined form reacts with the Folin uric acid reagent or its modifications (Benedict, 1922; Morris and MacLeod, 1922). Guillaumin (1922) states that the plasma contains mostly the free form and the corpuscles chiefly the combined; it has been suggested that part of this combined form is a combination with a pentose such as has been found in ox blood by Davis and Benedict (1921). Richter-Quittner (1921), however, states that uric acid is completely dialysable.

In experiments on the function of the kidneys, the simplest method, it might be thought, would be to use estimations made on the plasma. Apart altogether from the fact that the majority of the observations mentioned above have been made on man, while in these experiments it was proposed to use animals, Falta and Richter-Quittner (1921) have stated that, among other substances, urea, inorganic phosphate and sugar are not present in the corpuscles under physiological conditions, and do not enter them if precautions are taken to prevent injury to them when the blood is drawn.

Brinkman and Van Dam (1919) had shown previously that the corpuscles of frog and man are impermeable to glucose; and Ege (1921) has confirmed this conclusion for some animals, but not for man, and Van Creveld and Brinkman (1921) for man and rabbit. Apart from the discrepancies between the results of the different investigators, they also fail to agree as to what it is which causes the corpuscles to become permeable. Thus Brinkman holds that the commencement of clotting alters the permeability, while Falta states that *serum* has the same amount as plasma if defibrination is properly performed. The latter investigator also considers that corpuscles are permeable to sugar *in vivo*, since they use it as a food; but presumably they do not store it, or carry it, in the same way as plasma, and so contain none when blood is drawn with proper precautions.

If, therefore, under ordinary conditions of drawing blood into potassium oxalate, substances enter the corpuscles from the plasma, while *in vivo* they remain entirely in the latter, it would be necessary in these experiments to estimate them in whole blood, and with the aid of a hæmatocrit reading, calculate the amounts in the *plasma*, and so the amounts which are available for kidney secretion.

It seemed of interest, then, to inquire as to the distribution of these substances between plasma and corpuscles, and a series of experiments was undertaken with this object in view.*

METHODS.

Since the kidney concentration experiments were performed on cats, only the results obtained with these animals will be mentioned here.

The animals were anæsthetised with ether and bled from an artery through a paraffin-lined cannula, the blood being collected in paraffin-lined centrifuge tubes, containing the requisite amount of anti-coagulant; the sample (or a portion of it) was then immediately centrifuged. The estimations were performed on whole blood and plasma; the amounts in the corpuscles were calculated from hæmatocrit readings obtained from the same or parallel samples of blood.

The methods used for the analyses were:

For urea, creatinine and uric acid, the colorimetric methods of Folin (1921); the proteins of the plasma were precipitated by tungstic acid and the estimations performed on the filtrate: the urease method was used for urea, followed, in the plasma filtrate, by aspiration into standard acid and Nesslerisation, and, in urine, by direct Nesslerisation; the NH_3 in urine was estimated by aspiration into acid and Nesslerisation.

For inorganic phosphate the colorimetric method of Bell and Doisy (1920) was used, with the precautions suggested by Myers and Shevky (1921).

Special experiments were only done for the distribution of urea and creatinine; sometimes blood was taken at the end of an experiment in which two or more of the substances under investigation had been injected into the circulation and the distribution determined. Various substances were tried as anti-coagulants, especially sodium oxalate and sodium citrate, since Falta and Richter-Quittner (1921) state that they cause less damage to the corpuscles than potassium oxalate. Sodium citrate, however, was unsatisfactory, causing hæmolysis.

RESULTS.

The results obtained are set out in Table I.

These confirm, on the whole, the conclusions reached by previous investigators, and suggest that although there may be variations in the distribution of urea and creatinine in either direction, yet there is no regular departure from equality of distribution in either instance.

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* This piece of work was undertaken in conjunction with Dr. George Graham. With his permission I quote the results as far as concern the subject of this paper.

Inorganic phosphate, and possibly uric acid, show larger amounts in plasma than in corpuscles; this may have been because the conditions of experiment were somewhat different, these substances having been purposely injected into the blood-stream some time beforehand. Creatinine and urea appear to behave similarly after injection, as is seen in experiments No. 13 and 16.

TABLE I. *(PART I)*
DISTRIBUTION BETWEEN PLASMA AND CORPUSCLES.

EXPERIMENT No	ANTICOAGULANT	CORPUSCLES VOL %	BLOOD. mg PER 100 c.c.	PLASMA: mg PER 100 c.c. = P	CORPUSCLES. mg PER 100 c.c. = C (CALCULATED)	P/C
		UREA				
3	C	37	63·3	69 5	52·7	1·3
	B	38		70·4	51·6	1·4
4	A	42	62 5	66·9	56·4	1·2
	B	39		66·0	56·9	1·2
	C	37		67·7	53·8	1·25
5	C	35	50·9	52·2	48·6	1·1
[illegible]	C	37·5	84·3	86·2	81·1	1·1
7	C	33	39·9	36·8	46·4	0·8
12*	C	20	120	110	160	0·68
13*	C	29·5	101	103	96	1 07
16*	C	31	107	117	84 8	1·4
		CREATININE				
3	C	37	1 2	1·0	1 5	0·67
	B	38		[illegible]	1 4	0·78
4	A	42	1 2	1 1	1 3	0·85
	[illegible]	39		1 2	1 2	1·0
	C	37		1 2	1 2	1·0
12*	C	20	2·9	3·0	2·5	1·2
13*	C	29·5	1·9	2·4	0·7	3·4
16*	C	31	4·3	4·9	2·9	1·7
		INORGANIC PHOSPHATE				
12*	C	20	8·9	9·9	5 0	1·98
13*	C	29·5	7·7	8·5	5·8	1·5
16*	C	31	15·5	17·9	10·3	1·7
		URIC ACID				
13*	C	29 5	0·96	1·1	0·64	1·7

A = 0·1g SODIUM OXALATE PER 100 c.c. BLOOD
B = 1·0g SODIUM CITRATE " " "
C = 0·2g SODIUM OXALATE " " "
* SIGNIFIES THE SUBSTANCE INVESTIGATED HAD BEEN PREVIOUSLY INJECTED INTO THE BLOODSTREAM.

Iversen (1921) found that injected Na_2HPO_4 gradually entered the corpuscles of the rabbit, becoming organically combined, if excretion was prevented by nephrectomy. Theis and Benedict (1921) observed that uric acid added to blood remained in the plasma in 70 per cent. of the cases, in 30 per cent. becoming equally distributed. In the one experiment in which the uric acid distribution was determined, slightly more was present in the plasma as compared with the corpuscles. In my experiments it sometimes happened that the whole blood estimation was performed on blood, a portion of which was not subsequently centrifuged; in this way there might be an actual small variation between the amount originally present in the whole blood and plasma samples which would account for an apparent uneven distribution between plasma and corpuscles. This was sometimes unavoidable owing to the small quantities of blood available, and it is known that dilution of the blood occurs during a bleeding, the later samples being more dilute than the earlier.

These results, however, may not apply to blood *in vivo*, and attempts were made to estimate the urea in plasma obtained by centrifuging a ligatured vein (Van Creveld and Brinkman, 1921). The difficulties of estimation due to the small quantities of fluid obtained have so far prevented conclusive results being reached, but the figures, as far as they go, lend no support to the view that urea is exclusively present in the plasma.

In order to remove all uncertainty regarding the blood concentrations available for secretion, it was concluded that the estimations in the concentration ratio experiments had best be done on plasma; a few which were performed on whole blood, in which the assumption was made that there was equal distribution, gave results which agreed very well with the former.

SUMMARY.

(1) Urea and creatinine are evenly distributed between plasma and corpuscles in the blood of the cat.

(2) After the injection of urea and creatinine into the blood-stream the substances may be found equally distributed between plasma and corpuscles, or there may be a greater proportion in the plasma.

(3) After the injection of inorganic phosphate and uric acid a larger proportion is found in the plasma relative to the proportion present in the corpuscles.

REFERENCES.

BARNETT, G. D., AND ADDIS, T.—(1917) *J. Biol. Chem.*, **30**, 41.
BELL, R. D., AND DOISY, E. A.—(1920) *Ibid.*, **44**, 55.
BENEDICT, S. R.—(1922) *Ibid.*, **51**, 187.
BLOOR, W. R.—(1918) *Ibid.*, **36**, 49.
BRINKMAN, R., AND VAN DAM, E.—(1919) *Arch. Internat. Physiol.*, **15**, 105.
CATHCART, G. D.—(1916) *Biochem. J.*, **10**, 197.
CUSHNY, A. R.—(1917) 'The Secretion of the Urine,' London.—(1919) *J. Physiol.*, **53**, 391.
DAVIS, A. R., AND BENEDICT, S. R.—(1921) *J. Biol. Chem.*, **46**, Proc. v.
EGE, R.—(1921) *Biochem. Zeitschr.*, **111**, 189.
FALTA, W., AND RICHTER-QUITTNER, M.—(1921) *Ibid.*, **114**, 145.
FOLIN, O.—(1921) 'A Laboratory Manual of Biological Chemistry,' New York and London.
FOLIN, O., AND WU, H.—(1919) *J. Biol. Chem.*, **38**, 81.
GUILLAUMIN, CH. O.—(1922) *Compt. rend. Soc. de Biol.*, **86**, 258.
HUNTER, A., AND CAMPBELL, W. R.—(1918) *J. Biol. Chem.*, **33**, 169.
IVERSEN, P.—(1921) *Biochem. Zeitschr.*, **114**, 297.
JONES, W.—(1920) 'Nucleic Acids,' London.
MACLEAN, H.—(1921) *Brit. Med. J.*, **2**, 425.
MARSHALL, E. K., JUN., AND DAVIS, D. M.—(1914) *J. Biol. Chem.*, **18**, 53.
MORRIS, J. L., AND MACLEOD, A. G.—(1922) *Ibid.*, **50**, 65.
MYERS, B. A., AND SHEVKY, M. C.—(1921) *J. Lab. Clin. Med.*, **7**, 176.
RICHTER-QUITTNER, M.—(1921) *Biochem. Zeitschr.*, **124**, 106.
THEIS, R. C., AND BENEDICT, S. R.—(1921) *J. Lab. Clin. Med.*, **6**, 680.
VAN CREVELD, S., AND BRINKMAN, R.—(1921) *Biochem. Zeitschr.*, **119**, 165.
WILSON, D. W., AND PLASS, E. D.—(1917) *J. Biol. Chem.*, **29**, 413.
WU, H.—(1922) *Ibid.*, **51**, 21.
ZUCKER, T. F., AND GUTMAN, M. B.—(1922) *Proc. Soc. Exp. Med. Biol.*, **19**, 169.

VASCULAR CHANGES AFFECTING BULBAR NERVE CENTRES.

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CHANGES in the supply and hæmoglobin content of the blood cause variations in tone and excitability of bulbar centres which do not seem to have been systematically studied, though mentioned in the work of Bayliss (1920). The present work represents an attempt to investigate these effects in the dog.

METHODS.*

The right vagus only is cut. Stimulation of the cut nerve (central end) induces slowing of the heart with or without vaso-motor effects. Most cases chosen gave pure inhibitor response. The first record (Fig. 1) shows the type of reaction selected to work upon. The fall in blood-pressure is a cardio-inhibitor effect, for blocking of the left vagus stops it. Plethysmograph records of a limb also show that it is not a vaso-dilator effect. The strength of stimulus for eliciting this response varied, but was uniform for each series of observations. The blocking current, unless very strong, does not excite the vagus. With an ascending current from a cell with one volt or so difference of potential, blocking is complete on closure and recovery immediate on opening; the effect is the same if the current be reversed (kathode below).

The anæsthetic used was ether or C.E. mixture after a preliminary dose of morphia (6 mgr. per kg.). The anæsthetic was given either by artificial respiration by a trachea tube, or by natural respiration. The degree of anæsthesia was as uniform as possible throughout. The chief disturbing factor was change in the breathing. Occasionally artificial respiration was used to help revival. Measured quantities of blood were drawn by a cannula in the femoral artery. This was in some instances supplemented by clipping one carotid, the other carotid being connected with a manometer (Fig. 2). Reintroduction of the whipped blood by the jugular vein, as well as that of gum saline, was also studied.

Acting on the advice of Sir William Bayliss, to whom I am greatly indebted, I have used a 6·5 per cent. gum solution in all the later experiments, though at first only a little more than 5 per cent. was used, which had a viscosity between that of blood and that of serum. The 6·5 per cent. with its higher osmotic pressure and higher viscosity tends to maintain better a given pressure level.

It is believed that the tone of the cardio-inhibitor centre is depressed by hæmorrhage, and the warning to avoid this (Bainbridge, 1914) if one wishes to elicit facile reflex responses implies that this facility is a characteristic of tone, and may suggest that excitability and tone are synonymous. McWilliam (1900) in his work on chloroform effects on nerve centres discriminated between the two properties. Tone may be induced or increased reflexly in the cardio-inhibitor centre, *e.g.* by stimulating the central end of the vagus, as it may be depressed, *e.g.* by somatic nerves. The degree and duration of these artificially induced effects, as well as the natural state of tone, are taken in these experiments in conjunction with the immediate results of reflex excitation as a measure of the functional state of the centre under different conditions. They are considered under two heads—immediate and remote (see protocols). The duration of an effect indicates any changes of tonic activity in the cardio-inhibitor centre, while the promptness of the response and its extent (change of heart-rate) give an idea of reflex excitability. Blocking and unblocking also reveals the tonic condition.

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* Abbreviated at the request of the editors.

For investigating the effects of small hæmorrhages most cases chosen yielded a fairly well-marked slowing reaction to vagal stimulation (Fig. 3), and change of rate rather than of pressure has been the criterion—that is to say, that other centres are so slightly affected by small hæmorrhages as not to be worth investigation. When amounts of 50 per cent. or more of the total blood (on basis of 7 per cent. of body-weight) were rapidly bled, the effects were studied on cardio-inhibitory, vaso-motor and respiratory centres. Vaso-motor tone is measured by rise of pressure occurring spontaneously after block, and excitability by increased rise on stimulation of central end of vagus and sciatic.

A depressor vaso-motor effect has been rarely observed in these dog experiments to result from central vagus stimulation as described. Changes in the pressor response and tone are taken as the chief indication of the effects of bleeding on the vaso-motor centre. In testing the activity of the respiratory centre sciatic and vagus stimulation was employed. To the former the centre responds by strong inspiratory efforts.

RESULTS.

The protocols of some experiments are given, so as to reveal the method at a glance and to give the more important reactions. The procedure adopted is set out in the order of performance. Exp. III represents an exhaustive method carried out in some instances with extreme bleeding in a series of steps, re-bleeding after gum saline, and the stages of recovery with gum saline and blood contrasted. Before infusion in such cases the heart usually came to a stop, or showed extreme slowness and irregularity (Fig. 7) at or about the zero line of pressure. The amount of blood which could be drawn off before reaching this level is referred to as "the yield." The proportion of this amount to the calculated total varied from 60 to 85 per cent., according to rate of bleeding, condition, etc.

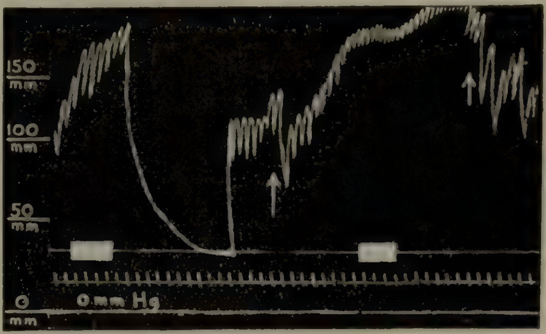

Fig. 1.—Well-marked reflex from cardio-inhibitor centre. It is annulled by blocking left vagus (first arrow). Sustained tonic activity shown when this is unblocked (second arrow). (Block by continuous current with difference of potential of about 1 volt.)

Jonescu (1900), with Bayliss, investigated the effects of asphyxia on the secretory powers of the salivary glands deprived of blood or oxygen, and noted a rapid decrease in secretion; the limits of the privation period consistent with recovery were not fully established. In the reaction from nerve centres investigated in the present experiments we possess a reliable key to tissue activity. The response is almost invariably the same to a given stimulus in uniform conditions over long periods. The rapidity is a great advantage as compared with secretion reaction as a test of tissue condition, that is, the condition resulting from mere blood changes on the one hand, and, to some slight extent, from the secondarily induced shock accompanying anæmia on the other.

Dolley (1909) has shown that nerve cells undergo morphological change from deprivation of blood, but in that case the duration period was not determined for the abolition of function.

EXPERIMENT I.—*Weight of Dog, 6 kg. Yield of Blood, 320 c.c.*

Nerve stimulation.	Amount bled.	Amount infused.	Left vagus.	Immediate effect of stimulus.	Latent period.	Heart rate.	Later rate.	Respiration.
	c.c.	c.c.			Secs.			
C.E.V.	—	—	Free	Fall B.P. and slowing	1·5	120–20	60	Inhib.
,,	150	—	,,	Ditto	2·5	120–50	100	,,
Sc.	—	—	,,	Rise B.P. accln.	1·5	100–130	100	Inc. rate and depth.
C.E.V.	150	—	,,	*Nil*	—	—	18 irreg.	Expy. efforts.
Sc.	—	—	,,	Slight rise	2	*Nil*	Irreg.	,, ,,
C.E.V.	—	150 g.s.	,,	Rise B.P., later slowing	1·5 for rise	110–80	100	Inhib.
,,	—	150 g.s.	,,	Slowing	1·5	110–80	100	,,
,,	200	—	,,	*Nil*	—	—	120	Slight inhib.
,,	—	300 blood	,,	Slowing	1	120–60	70	Inhib.
,,	—	—	Blocked	Rise B.P.	1·5	—	120	,,

EXPERIMENT II.—*Weight of Dog, 14 kg. Yield of Blood, 875 c.c.*

C.E.V.	—	—	Free	Slowing fall B.P.	1	150–0	100	Inhib.
,,	275	—	,,	,, ,,	1·5	200–0	150	,,
,,	150	—	,,	,, ,,	2	150–50	120	,,
Sc.	—	—	,,	Rise B.P. accel.	1·5	150–180	180	Inc. rate depth.
C.E.V.	100	—	,,	Slowing	3	180–100	140	Inhib.
,,	150	—	,,	Rise B.P.	1·5	*Nil*	150	,,
,,	150	—	,,	*Nil*	—	,,	Irreg.	,,
,,	—	—	Blocked	,,	—	—	Irreg.	Slight inhib.
,,	50	—	Free	,,	—	—	Stopped	Expy. efforts stop in expn.
,,	—	700 g.s.	,,	Slowing	2·5	140–10	50	Inhib. after recovery.
,,	—	—	Blocked	Rise B.P.	2	*Nil*	120	Inhib.

EXPERIMENT III.—*Weight of Dog, 8 kg. Yield of Blood, 500 c.c.*

C.E.V.	—	—	Free	Slowing fall B.P.	1·5	120–60	80	Inhib.
,,	150	—	,,	,, ,,	2	140–80	130	,,
,,	50	—	,,	,, ,,	2	150–100	120	,,
,,	50	—	,,	,, ,,	2·5	180–160	170	,,
,,	100	—	,,	Falls B.P.	—	*Nil*	160	,,
,,	—	—	Blocked	Rise B.P.	2	,,	160	,,
,,	100	—	Free	*Nil*	—	,,	100	*Nil.*
Sc.	—	—	,,	,,	—	,,	100	Slight increase.
,,	—	—	Blocked	,,	—	—	100	,, ,,
C.E.V.	—	—	,,	,,	—	—	—	Inhib. slight.
,,	50	—	Free	*Nil* (zero pressure)	—	—	*Nil*	Expy. stop *nil.*
,,	—	170 g.s.	,,	*Nil* (20 mm. Hg.)	—	—	100 irreg.	*Nil* (stopped).
,,	—	150 g.s.	,,	Slowing	4	120–100	120	Inhib.
,,	—	200 g.s.	,,	,,	2	150–90	130	,,
Sc.	—	—	,,	Rise B.P.	2	130–150	150	Increased.
,,	450 (2nd Bleeding)	—	,,	*Nil*	—	—	*Nil*	Stop slight exp.
C.E.V.	—	400 blood	,,	Slowing	1·5	130–60	80	Inhib.
,,	—	—	Blocked	Rise B.P. accel.	1·5	120–140	100	,,
Sc.	—	—	Free	Rise B.P.	2	*Nil*	120	Increased.

We may consider the results in relation to each of the three centres:

(1) *Cardio-inhibitor.*—The animals included in the Table gave a more or less marked slowing of beat as a response when the central end of the cut vagus was stimulated. It will be noticed that bleeding up to 30 or 40 per cent. of the total yield, and up to 50 per cent. of the calculated total, leaves the reflex response but slightly affected, though it disappears generally when this limit is passed. Gesell (1918) found in some cases, for the respiratory and CO_2 -curves, great resistance up to a certain degree of hæmorrhage, and then a sudden break. The loss of cardio-inhibitor reflexes is, as a rule, sudden, rarely progressive. The recovery process after infusion is more progressive. Occluding the carotid artery is a much more effective procedure in suppressing the inhibitory reflex than is bleeding of moderate degree (Fig. 2). Effects frequently noted from moderate bleeding were the lengthening of the latent period (Fig. 3) of the reflex inhibitory response and the diminution of its duration; the latent period decreases again with recovery (Fig. 4). Activity of the cardio-inhibitor centre does not depend on a given arterial pressure; in slow bleeding responses may persist down to a pressure of 50 mm. Hg. or less, and even if abolished may return again at low pressures. Clipping the carotid artery interferes greatly with the blood supply, and the cardio-inhibitor centre fails quickly; yet the vaso-motor centre maintains a considerable degree of activity under this condition. It thus affords a contrast between them. There is no important disturbance of bulbar circulation by occlusion of one carotid and one jugular. This has been remarked already (Roberts, 1921). Respiration, Roberts says, is not influenced by clipping the artery of the other side in addition, the only result of this being a slight rise, of mechanical origin, in the blood-pressure. But a disturbance of bulbar reactions results from this occlusion. The most obvious of them is the failure of (a previously well-marked) response of the cardio-inhibitor centre; this change I have found to be invariable; there is also increased heart rate, which shows diminished tone of the centre.

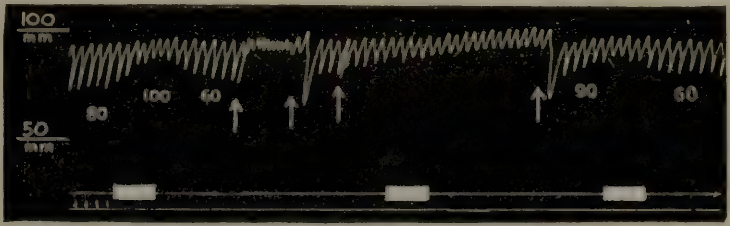

FIG. 2.—Inhibitor reflex of slight degree abolished by occlusion of carotid artery between second pair of arrows and return of it when the artery is free.

Gesell (1918) concluded that a great reduction of volume flow may occur with a constant head of pressure; this flow may, with change of pressure, remain constant or change in either direction. As seen by the uniform reactions of bulbar centres, especially the cardio-inhibitor, the flow to them seems to remain uniform, or at any rate efficient, at low pressures, except after rapid bleeding, when it is disturbed for a time. It is also disturbed by clipping the second carotid. The reduction in heart-rate from central vagus stimulation after withdrawal of 40 per cent. of the calculated total blood (300 c.c. in an animal of 10 kilos), and even up to 70 per cent., sometimes may be nearly as great as prior to bleeding, yet the moment the artery is clipped the response is abolished. The cutting off of the direct supply by the carotid evidently greatly hampers the circulation in the centre, even though both vertebrals are free. The duration of arterial occlusion also affects that of the reflexly induced tone in the centre. After removal of the clips the pulse-rate tends to be re-established rapidly when stimulation ceases, though the reflex response of slowing may have been immediate. It shows distinctly the difference between the properties of reflex excitability and tonic activity as affected by oxygen want. Bleeding first affects the latent period and the duration of reflex tone.

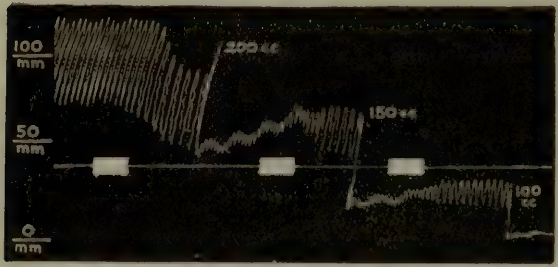

Fig. 3.—Prolongation of latent period of cardio-inhibitor reflex after bleeding.

The withdrawal of small amounts of blood such as may represent that lost in hurried dissection has little effect in altering the condition of the cardio-inhibitor centre. A more probable cause of such alteration is rough handling of the tissues. I have frequently noticed that when the carotid sheath was stretched, dragged upon and torn rapidly open, the responses of the centre, either to vagus or sciatic stimulation, were not well marked. Shock of this sort is distinctly deleterious to the centres. When the sheath is dissected with care and the general handling of the tissues is gentle, the reflex excitability of the vagus centre and the facility of inducing tone in it are much better for a given degree of anæsthesia. In some instances it was noticed that the latent period of the slowing was very long (5 to 10 seconds) even before bleeding; moderate bleeding more quickly abolishes the reflex in such cases (Fig. 5). Bayliss (1918) and others ascribe the chief symptoms of shock to absorption from tissues injured, but do not altogether discard reflex effects on the centres. I think this latter factor comes in, at any rate in neck dissection with tissue abuse, in affecting the reactions of bulbar centres. Owing to restriction of space only a few records illustrating the protocols are inserted, but the more important results are explained by these. Fig. 4 represents a condition reached as follows: The animal (8 kg.) was bled to the extent of 600 c.c.; gum saline infusion was carried out and recovery of the reflex slowing with the standard stimulus occurred. The animal was again bled. The heart had now become very slow

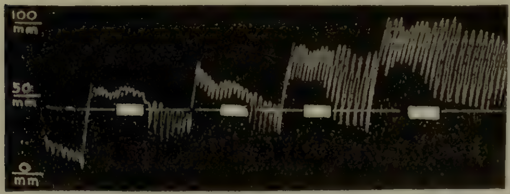

Fig. 4.—Recovery of inhibitor reflex with gradual shortening of the latent period. 100 c.c. blood infused at each stop. Dog 10 kg.

and irregular after the second bleeding, and a little more gum saline was introduced which brought it to the condition of the first step shown in Fig. 4 (B.P. about 15 mm. Hg.) before infusion with blood. At this stage the C.E.V. stimulation gave no response; as blood was infused the response reappeared and improved rapidly—more rapidly than with gum saline. The restorative effect of gum saline, when the heart is in very poor condition and the centres are exhausted, is not quite so good as that of blood. Fig. 6 shows practically no recovery of the inhibitor response with gum saline in such a condition. Fig. 7 is from a similar instance (high yield and secondary bleeding); gum saline is not equal to blood in effecting recovery either of tone or excitability. The necessary hæmoglobin being added, in this case recovery was prompt. The privation of the centres was extreme and the hæmoglobin limit passed. It will be referred to again under vaso-motor effects.

(2) *Vaso-motor.*—In most cases, after full yield of blood, all three centres showed good recovery with gum saline alone, but when one centre failed in such circumstances it was generally the vaso-motor. Vitality, although without regularity, may still be shown by the respiratory centre, though perhaps without possibility of ultimate full recovery. The vaso-motor centre is judged to fail by reason of loss, not of reflex response, but of tone. Blood sometimes remedies this failure when gum saline cannot, when the yield has been very high. The rise of pressure after clipping the second carotid artery seems to me to be somewhat greater than that attributable to mere mechanical effects. There is a small but continued rise, and when the clip is removed the sudden drop is greater than the immediate rise on putting it on. After moderate bleeding there is also a steady rise of pressure. The centre becomes more active either as a result of volume change or oxygen want. It is also more responsive to reflex excitation in these circumstances. The vaso-motor centre seems to be excited by the disturbance of the clip in the opposite direction to the effect on the inhibitor centre. The vaso-motor centre remains active—shows tone and excitability—for some time after the cardio-inhibitor properties are lost in progressive bleeding. Its tonic functions are quickly suppressed at low pressures before excitability goes—the centre can respond to a stimulus but the effect passes off quickly.

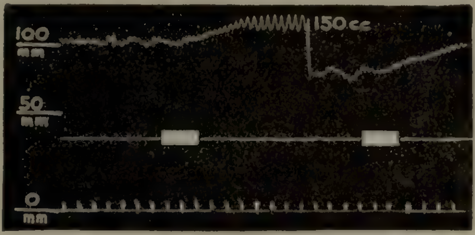

FIG. 5.—Inhibitor reflex with long latent period abolished by moderate bleeding—20 per cent. of total.

Bayliss has remarked that it is useless to employ transfusion or gum infusion if the vaso-motor centre vitality be lost. His observation that it is impossible, in such a condition, to raise the pressure beyond a low level, except temporarily, even by injection of large volumes, has been verified in these experiments (Fig. 6). The record shows in the different steps of gum saline infusion that there is no response from the centres. There had been some half hour's exposure to low gum saline pressure, but in a poor type of animal. The pressure rose to about 100 mm. Hg. as a maximum, but it fell away quickly when infusion ceased. Blood was immediately tried to replace gum saline, but with no better result. But in other cases of severe bleeding blood was more effective than gum. In similar cases where the recovery of pressure likewise failed with gum saline, the cardio-inhibitor centre was shown to be still active—reflexly excitable and active in tone (Fig. 8). The survival powers of vaso-motor and cardio-inhibitor centres present somewhat anomalous relations. In several instances the latter reacted when the former remained inactive. The cardio-inhibitor response goes more rapidly than the vaso-motor, in rapid bleeding, but with gum saline infusion it returns more readily than the vaso-motor response. In Fig. 8 the cardio-inhibitor centre responds well to stimulation but the vaso-motor centre does not. In all the animals whose blood-pressure was easy to restore there was generally a ready return of reflex response in both centres. Death of the vaso-motor centre means failure of the necessary circulation pressure, except for brief periods. A remnant of vaso-motor vitality, on the contrary, means recovery if the respiratory centre be not too far gone; but this recovery is often not full, whereas the cardio-inhibitor recovery is frequently so. Vitality of the vaso-motor centre is apparent when the reflex response is recovered, but a loss of reflex does not mean death, though the margin between the excitability threshold and absolute failure seems to be small. It sometimes recovers late (Fig. 9, end of tracing). With the cardio-inhibitor centre, on the other hand, reflex excitability and tone, as elicited through the vagus, may be suppressed over long periods and yet the centre may show eventual recovery. Its reflex reactions appear to succumb more quickly than those of the vaso-motor centre, but its vitality and resistance are really greater (Fig. 8). In some instances, if bleeding be slow, quite a good response is given by the cardio-inhibitor centre down to pressures of 30 to 40 mm. Hg. These cases recover very well with gum saline, the vaso-motor and respiratory centre also often recovering if exposure to low pressures has not been too prolonged. In Fig. 8 there is every sign of death of the vaso-motor centre—no response to sciatic or vagus excitation and no tone—but the inhibitor centre is quite active.

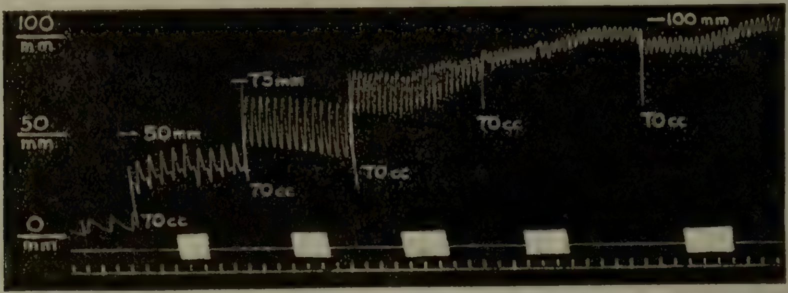

Fig. 6.—Suppression of function in both vaso-motor and cardio-inhibitor centres after severe bleeding and exposure. Infusion of gum saline.

We sometimes see a difference between the effects of gum saline and blood as restoratives; with equal amounts a lower pressure is established by the former, though restoration of reflex function, both in vaso-motor and cardio-inhibitor centres, may be quite good. The vaso-motor centre, however, must be defective in tone in such cases, though its excitability be good. The all-important factor, as Bayliss (1918) points out, is the oxygen carrier, and it is certainly so, for tonic and permanent recovery. The tonic activity of the centres seems to depend more on a due supply of oxygen than the reflex excitability, which may still be exhibited in pronounced oxygen want. Blood infusion or transfusion is the desideratum in cases of severe hæmorrhage in human beings, but gum saline, while effective as a permanent agent in cases with a sufficient hæmoglobin remnant, should not be lost sight of as a temporary expedient in still more serious loss.

(3) *Respiratory.*—Roberts (1921) says the respiration ceases on sudden free hæmorrhage from the aorta, because of anæmia of the medulla. Anæmia of the medulla brought about slowly in the dog does not always stop respiration. For instance, in an animal of 6 kilos 320 c.c. were drawn off in lots of 70 to 80 c.c., at intervals of two to three minutes; the respirations, after some increase of rate in the early stages of bleeding, were reduced to 4 or 5 per minute, but remained regular. They increased to 10 per minute after gum saline. Roberts got full stoppage of respiration by clamping all four arteries to the brain, but, with a supply from one vessel only, the respirations remained quite frequent and regular. The respiratory centre resists change in volume flow very well up to a certain point. Death of the centre, as a rule, quickly supervenes once this point is passed.

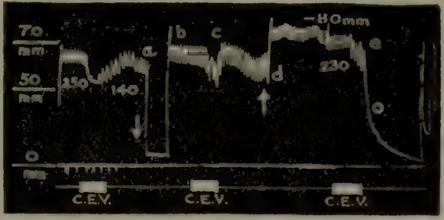

FIG. 7.—Bleeding and re-bleeding; second "bleeding" of gum saline and reintroduction of gum saline shown at *a* and *b*. There is no inhibitory response. But at *d* some 150 c.c. of blood were run in and the cardio-inhibitory centre recovered function (third signal).

The respiratory centre responds well to sciatic stimulation to the very end. When near exhaustion this response may take on an active expiratory character. This expiratory response is also usually elicited at this stage by stimulation of the central end of the vagus, which normally causes apnœa with all strengths of current. In this condition, too, even the weak blocking current sets up some expiratory efforts, the expiratory part of the centre being evidently very sensitive.

Doubt still exists as to the relation between the respiratory and cardio-inhibitor centres. In these experiments no overflow was found from the former to the latter; this confirms the observations of Bainbridge (1920). In some dogs respiration is normally very slow. Even morphia, which generally causes panting, causes little or no increase of breathing in these, and the centre may display a great amount of resistance, and is especially prone to exhibit the expiratory response above referred to.

Bayliss (1920) has drawn attention to the fact that the respiratory is more sensitive than the vaso-motor centre. But the respiratory centre may be active, with gasping respirations, or even regular breathing, at a time when all response is abolished in the vaso-motor and cardio-inhibitor centres—for instance in profuse rapid bleeding with or without administration of gum saline.

Bayliss considered the gasping condition of the respiratory centre as a state or sign of failure in which the heart is insufficiently supplied with oxygen. That is certainly so. The present work merely shows that by prompt action the centre may be restored from an apparently hopeless condition. In ultimate survival, which evidently Bayliss means, it is different in this respect: the respiratory is not at all so resistant as the cardio-inhibitor centre. While its reflex or automatic activity may be exhibited when all other reflexes and tonic activity are suppressed, and when it is in a dying condition, it may fail just as these are being revived in the cardio-inhibitor centre; and it may fail even when the vaso-motor centre is active (this requiring artificial respiration for its demonstration), but in a majority of the present experiments the vaso-motor centre died before the respiratory centre. For that reason alone I place the vitality of the respiratory centre as somewhat higher than that of the vaso-motor centre.

The respiratory centre, having become inactive from oxygen want, cannot recover with gum-saline alone, although the heart beats, the inhibitor centre responds and a pressure of 70 or 80 mm. Hg. is reached. In this condition I have frequently brought about recovery of the respiratory centre (Fig. 10) by the addition of blood to the extent of 10 per cent. of corpuscles. The corpuscles were measured roughly by centrifuge (1/10) and more accurately with hæmocytometer (1,000,000 to 1,100,000 per c.mm.), as there was none of the clumping described by Bayliss (1920) for cats. In some of these cases there was no restoration of function in the vaso-motor centre. Prolonged expiratory efforts usually mark the close of activity in the respiratory centre. When the centre is failing a sciatic stimulus remains effective in producing the well-known increase in activity which affects both phases, but, as usual, chiefly the inspiratory one. A rise of blood-pressure accompanies this increased ventilation, which is in part mechanical and in part due to a feeble response of the vaso-motor centre. In most instances the mechanical effect alone occurs as the vaso-motor centre is not responsive.

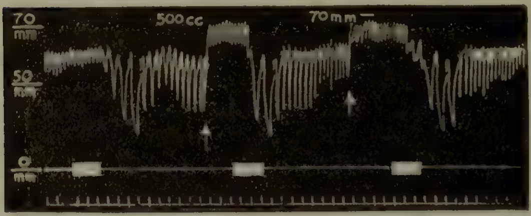

FIG. 8.—Good recovery shown in the inhibitor centre with complete failure of the vaso-motor centre. Natural respiration. The pressure never rose beyond 70 mm. Hg. and there was no reflex rise on sciatic stimulation—(not shown).

DISCUSSION OF RESULTS.

In placing the cardio-inhibitor centre first in order of temporary failure, account is taken of the increased length of the latent period, the well-marked effect of clipping the artery, the diminished tone effect and that of sudden bleeding. The order of suppression (temporary) of function in these bulbar centres is different from that of their irretrievable abolition. Thus:

Temporary suppression.	*Abolition.*
(1) Cardio-inhibitor.	(1) Vaso-motor.
(2) Vaso-motor.	(2) Respiratory.
(3) Respiratory.	(3) Cardio-inhibitor.

The order means that while the respiratory centre holds out after function has been suppressed in the other two, yet it is more liable to suffer permanent damage than the cardio-inhibitor centre, if we take into account their recuperative powers. The relative positions of the vaso-motor and respiratory centres are somewhat doubtful. In some cases the vaso-motor centre resists apparently better than the respiratory, but, since in so many experiments the former succumbed first, I think it is the more vulnerable. As the respiratory centre is failing, it becomes less responsive to the inhibition produced by vagus stimulus, though even more sensitive to sciatic stimulation, which produces increased inspiratory efforts. One notes the diminished vagal inhibitory response at a time when the cardio-inhibitor centre is still quite active. The centre later still shows the expiratory type of response, already mentioned, to vagus stimulation. The increased CO_2 in the blood with the accompanying diminution of pH makes the centre extremely sensitive, as pointed out by Haldane and Priestly (1905). This increased sensitiveness connotes a greater tendency to escape from inhibition. There is practically no response in the dog upon which one may count with more certainty than that of respiratory inhibition to central vagus stimulation, of all degrees of intensity, but it becomes diminished as the sensitiveness of the centre increases in another direction. In the dog respiration is generally slow (12 to 15 per minute) when the panting has passed off after a morphia injection. In the early stages of hæmorrhage it increases in rate slightly; later, at a time when the centre is very sensitive, it becomes very deep, and is gradually reduced in rate in further privation. As Bayliss (1918) points out, quick and shallow respiration does not result in more effective ventilation. I have never seen the rapid shallow respiration described as accompanying some forms of shock and acidosis in these cases of simple bleeding. Bayliss explains the shallow breathing by a modification of the Hering-Breuer reflex in which the inhibitory response of the centre would be exaggerated. The respiratory centre in the cat shows some difference from that of the dog, *e.g.* in reacting to a tracheal plug; this is possibly another. The inhibitor effect of C.E.V. stimulation is certainly diminished in the dog with failing centre when it seems to be increased in the cat.

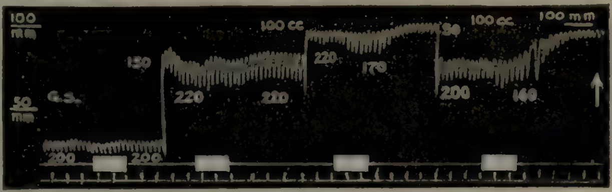

FIG. 9.—Slow recovery of cardio-inhibitory centre functions. Vaso-motor centre (tested at stops) functionless till near the end. Note that the pressure has actually fallen with last portion of the infusion.

In reference to the superiority of blood over gum saline in recovery, it must not be taken as contradicting the contention of Bayliss (1922[1]), that gum saline was quite as effective as blood. He always assumes that sufficient hæmoglobin is left to carry on oxygenation, whereas in most of my experiments where the opposite is claimed, that reserve was passed. The quick return of the inhibitor response (Fig. 7) is a case in point. The necessary hæmoglobin here quickly effected what the gum saline failed to do. Though I have sometimes seen an apparently quicker recovery of function with blood, for less severe bleeding, yet in general, as Bayliss remarks, it is surprising what gum saline can do in presence of small amounts of hæmoglobin. Where it is simply a question of increasing the volume of fluid to the tissues, then if the hæmoglobin content is adequate, gum saline serves as well as blood for the overcoming of stasis and reduced alkaline reserve resulting from deficient oxidation, and must be considered an invaluable agent in such cases. Where the hæmoglobin content is defective, gum saline can only produce a temporary though sometimes striking return of activity in the centres. Gum, I think, hastens clotting. I have not seen this effect referred to. It seems a salutary one, though very troublesome in experimental work of this sort. Sir William Bayliss suggests to me that it may be due to the calcium of the gum.

The total amount of blood which may be removed, before the pressure falls to the zero line and the heart-beat fails, is variable. Sometimes the heart becomes irregular after a loss of 60 per cent. of the calculated total, if bled rapidly; with slow bleeding, in steps, the loss may proceed to 80 per cent., or more, of the total. The condition of the animal and the time that it has been under experiment also affect the result. Animals rapidly bled to a state of great slowness and irregularity of the heart show good recovery with gum saline infusion if not delayed.

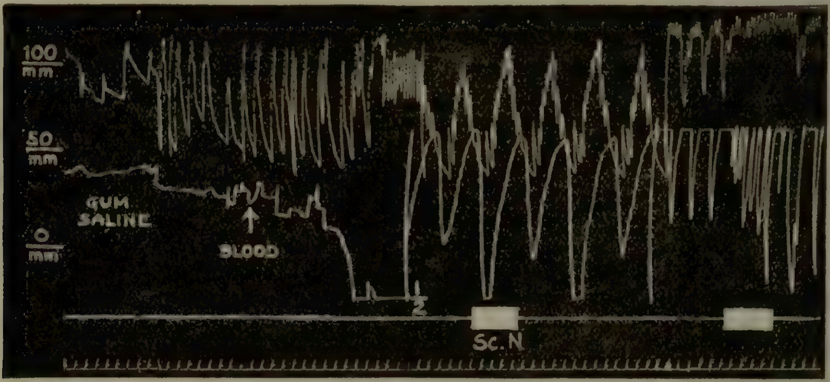

Fig. 10.—Failure of respiratory centre with gum saline (20 minutes at low pressure). Return of activity with blood (begun at arrow). Delayed return of reflex to sciatic stimulation (second signal). No response, first signal. Upper tracing = blood-pressure; lower tracing = respiration; down-stroke represents inspiration.

Bayliss ascertained the time-limit for recovery of bulbar centres in cats exposed to low blood-pressure of 58–60 mm. Hg. to be as long as 1½ hours or more. In the present work the time-limit was investigated in the case of still lower pressures than those employed by Bayliss. After full yield of blood to heart-stop and zero-pressure a little gum saline was introduced to keep the heart going at some 30 mm. Hg., and it was found that the centres sometimes recovered after half an hour under these conditions. This corresponds fairly well with the results of Richet and his associates (1918) for recovery in dogs. Stewart, Guthrie, Burns and Pike (1906), who deprived the medulla of blood by occlusion of vessels, found varying intervals for recovery after complete anæmia.

They considered half an hour exceptionally long. The condition so produced must differ in some important respects from that due to hæmorrhage.

Full recovery in the respiratory and vaso-motor centres after more than fifteen or twenty minutes was rare. There is a difference in the efficiency of a pure gum saline solution and the same plus a little blood in the survival of the starved centres in such circumstances. Athanasiu and I (1922), working at the Marey Institute, found that survival in the nerve centres of dogs, as exhibited by the electrical variations, occurred with artificial circulation from the heart-lung preparation of a second dog some twenty minutes after the centres had been drained of blood, and after more than an hour of shock from the very severe operation of exposing cerebrum, medulla, cord and nerves. Prior to the irrigation the animal was fully bled.

The duration of the severe anæmia consistent with good recovery after total bleeding does not exceed half an hour for the vaso-motor and inhibitor centres, more for the latter than the former. If the time were longer than this, recovery in vaso-motor and respiratory centres did not take place at all, and the heart-beat, with re-establishment of low pressures, recovered only temporarily; in some of these only the cardio-inhibitor centre remained responsive. The respiratory centre and the heart-beat, not the pressure, afford the indications for the amount of gum saline to be employed during the privation period. Both keep active sometimes at very low pressures which maintain a fixed level with or without a little extra gum saline. Complete privation, that is, when heart-stop and zero pressure result, cannot be supported for more than a few minutes. But even in such cases, if the yield is small, the heart may resume its beat spontaneously, and in these it is only by secondary bleeding, after the introduction of some gum saline, that one can reach a very low blood content.

Bayliss (1922[2]) has calculated for cats that 70 per cent. of the total blood is replaceable by gum saline, consistently with complete recovery. The fluid then would contain about 12 volumes per cent. of corpuscles. Henderson and Haggard (1922) removed 80 per cent. of the blood (on a basis of 7·3 per cent.) and got recovery with gum. Bayliss (1922[2]) thinks their percentage must have been larger, *i.e.* working on a lower basis. In my experiments there is no question of lasting recovery, but only of a temporary restoration of function, with gum saline. More than 70 per cent. of blood was removed, on whatever basis we work, yet function in nerve-centres was re-established with gum saline. The degree of hæmorrhage compatible with recovery of function was extreme, but accurate measurements of the duration of this privation period could not be made. Certainly, in some instances, such as that of No. 2, a period of half an hour elapsed from the end of a second "bleeding" to the beginning of infusion. The fluid that came away in the second bleeding was generally but light red in colour, and contained 7 or 8 per cent. by volume of corpuscles.

CONCLUSIONS.

The reflex excitability of bulbar nerve centres differs from their tonic activity, whether natural or induced by reflex excitation. Bleeding up to 40 or 50 per cent. of the calculated total blood affects the first-mentioned less than the second, though both properties depend on a proper blood supply. The vaso-motor centre is less rapidly affected by change of volume flow and oxygen content than is the cardio-inhibitor centre. Both function after excessive bleeding and infusion of gum saline, until the hæmoglobin is reduced to about one-fifth of the normal amount (80 per cent. of the total blood removed). Loss of blood amounting to 85 or 90 per cent. and brought about by secondary bleeding was not sufficient to cause death of the centres, if not too long exposed to these conditions. The vaso-motor centre in these extreme conditions is the most susceptible, though there is little difference between it and the respiratory centre. The cardio-inhibitor centre, though the earliest to suffer, is the most resistant to irreparable damage.

Rough handling of the tissues in dissection is a more serious cause of disturbance of reflex responses in the nerve centres than moderate amount of hæmorrhage.

Gum saline effects recovery of the centres after long periods of exposure to very low hæmoglobin content, represented by about 7 or 8 per cent. by volume of corpuscles. For permanent recovery blood is needed in addition for these extreme cases ; in lesser degrees of privation, to 12 to 15 per cent. of corpuscles, gum saline alone rapidly effects recovery.

REFERENCES.

ATHANASIU AND BARRY.—(1922) *Compt. rend. Soc. de Biol.*, **87**, 341.
BAINBRIDGE, F. A.—(1914) *J. Physiol.*, **48**, 332.—(1920) *Ibid.*, **54**, 3.
BAYLISS, W. M.—(1918) 'Intravenous Injection in Wound Shock,' London (Longmans, Green & Co.).—(1920) *J. Pharm. & Exper. Therap.*, **15**, 29.—(1922)[1] *J. Amer. Med. Assoc.*, **78**, 1885.—(1922)[2] *J. Physiol.*, **56**, p. xlvi.
DOLLEY, D. H.—(1909) *J. Med. Res.*, **21**, 95.
GESELL, R.—(1918) *Amer. J. Physiol.*, **47**, 489.
HALDANE, J. S., AND PRIESTLEY, J. G.—(1905) *J. Physiol.*, **32**, 225.
HENDERSON, Y., AND HAGGARD, H. W.—(1922) *J. Amer. Med. Assoc.*, **78**, 697.
JONESCU.—(1909) *Arch. Internat. Physiol.*, **8**, 59.
MCWILLIAM, J. F.—(1900) *J. Physiol.*, **25**, 262.
RICHET, C., BRODIN, P., AND SAINT-GIRONS, F.—(1918) *Compt. rend. Acad. des Sci.*, **167**, 618.
ROBERTS, F.—(1921) *Idem.*, **55**, 346.
STEWART, G. N., GUTHRIE, C. C., BURNS, R. L., AND PIKE, F. H.—(1906) *J. Exper. Med.*, **8**, 289.

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OF

EXPERIMENTAL PATHOLOGY

THE EXCRETION OF "ACETONE" AND NITROGEN IN NAUSEA AND VOMITING OF PREGNANCY.

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THERE have been many theories of the ætiology of the nausea and vomiting of pregnancy, the great majority of which have been based upon clinical observation, and which observations, on the whole, have remained unconfirmed. Of the possible causes to which has been ascribed the origin of this condition, there only remains a certain amount of evidence in favour of a neurosis, or intestinal intoxication, or some metabolic perversion. Observations based upon scientific measurements have been very few, and such as have been made have gone decidedly to discredit most of the theories of the ætiology based upon ordinary clinical findings. In our opinion, however, three contributions stand out prominently, though the general results have led to a negative rather than a positive position.

The first of these was in 1910 by Underhill and Rand (1910). By means of a vigorous criticism of the older work they showed very clearly that it was possible to ascribe all the changes which are to be found in the nitrogen partition of the urine in pernicious vomiting to starvation, and that such alterations as had been observed became, or tended to become, normal by the administration of carbohydrate. They thus ascribed the changes found in the urinary picture to mere lack of carbohydrate due to the starvation of necessity accompanying the vomiting. Though their results were criticised, nevertheless their contention would seem to be established. Losee and Van Slyke (1917) in a general paper on toxæmias of pregnancy published a series of facts upon pernicious vomiting, which showed that this condition was not accompanied by any excess of amino-acids, either in the blood or in the urine, nor that, if acidosis were measured in terms of the bicarbonate reserve of the body, was any true acidosis to be observed. They did, however, confirm the finding of high ammonia coefficients and low values for urea in the urine. Though they considered these findings to have some diagnostic value, they did not attempt to put any interpretation upon them. Later, Gilliatt and Kennaway (1918), from a study of two cases of pernicious vomiting, were of the opinion that although starvation might perhaps account for some of the changes observed in the urine, this alone was not sufficient. They pointed out that the ammonia coefficient, or, as they expressed it, the ammonia index, might rise very rapidly in pernicious vomiting, whereas such sudden increases were not usual in starvation, and that this coincided with the production of large amounts of acetone bodies; also that after abortion the ammonia index fell with almost equal rapidity. They also drew attention to the scarcity of data on the part played by the acetone bodies in pernicious vomiting.

The general conclusion which could be drawn from these contributions would be that the metabolic disturbance accompanying this condition is of the same essential nature as the disturbance caused by starvation, but is much more intense, although it never reaches such an intensity as to produce any marked disturbance of the acid-base equilibrium.

At about the same time as the publication of the paper of Gilliatt and Kennaway, a theory of the origin of the nausea and vomiting of pregnancy, based upon an essential carbohydrate disturbance, was put forward, which theory was afterwards amplified, and supported by a large mass of clinical observation (Duncan and Harding, 1918; Harding, 1921; Harding and Watson, 1922). The present paper deals with a series of exact measurements upon the urines of a number of cases of pernicious vomiting, taken from the wards of the Toronto General Hospital. The cases range from moderate to severe in type. All were under the direct clinical observation of one of us, and all were treated in essentially the same manner. This treatment in general consisted of rest with isolation, of the administration of carbohydrate to the amount of 50–100 gm. per day, usually by rectum, and the giving of fluids either by mouth (if it were possible for the patient to take them by that route), or interstitially or intravenously. The more detailed management of such cases is to be found in a previous paper. These observations, then, have the advantage of being made on patients who were under a unified control, both from a clinical and a scientific standpoint. We determined the daily excretion of total acetone bodies, total nitrogen, ammonia and urea.* We also determined the total acetone bodies in the blood, and looked for any evidence of "acidosis," by a determination of the CO_2-content of the alveolar air.

Case I (R—).

Primipara, aged 23; mild case of occasional vomiting for about two months. Previous history unimportant, though described as of a neurotic type by home physician. Physical examination normal. Patient was able to take small amounts of food and to drink water on entering hospital, consequently all that was advised was rest in bed with isolation and light carbohydrate meals. Recovery uneventful.

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* Analytical methods: Acetone, aceto-acetic acid and β-oxybutyric acid by combined method of Van Slyke, and results expressed as total acetone (Van Slyke, 1917; Van Slyke and Fitz, 1917); total nitrogen by method of Kjeldahl-Gunning; ammonia and urea by urease method of Van Slyke and Cullen (1914).

TABLE I.—*Case I (R—) : Urine Analysis.*

Total acetone in blood ·093 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Dec. 6	525	3·4	·5	2·5	1·3	Light carbohydrate meals.
,, 7	400	4·1	·8	2·4	1·2	,, ,,
,, 8	820	4·3	·6	3·0	·3	,, ,,
,, 9	860	3·4	·4	2·5	·1	Protein in diet.
,, 10	350	1·3	·1	1·0	—	,,
,, 11	730	3·1	·3	1·3	—	,,
,, 12	650	1·9	·1	1·5	—	,,

Case II (N—).

Primipara, aged 28; two months pregnant. Nauseated a month; a little vomiting during the previous week. Vomiting then became severe. No food two days previous to admission to hospital. History of biliousness at menstrual periods. Well nourished; negative physical findings, except epigastric pain on pressure. Treatment: Isolation; nutrient enemata, 10 per cent. glucose, 6 oz. *t.i.d.*; fluids urged by mouth. Rapid recovery, and after-history uneventful.

TABLE II.—*Case II (N—) : Urine Analysis.*

Total acetone in blood ·239 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Nov. 24	500	5·0	·5	3·7	4·7	—
,, 25	660	8·0	1·1	5·9	3·5	—
,, 26	755	8·1	1·1	5·7	1·2	Carbohydrate meals.
,, 27	525	5·1	·7	3·2	0·1	,,
,, 28	750	5·2	·6	2·8	—	Protein in diet.

Case III (Br—).

Multipara, aged 24. First pregnancy terminated by operation at 7 or 8 months. Second spontaneously aborted at 3 months. Present pregnancy about 3 months. Vomiting started about four weeks before admission to hospital as morning sickness, and had increased. Well nourished, obese woman, rather excitable. Physical examination negative. Treatment: Nutrient enemata, 10 per cent. glucose in saline of 6 oz. *t.i.d.*; bromide gr. xx, chloral gr. x were added to evening enema. Weak tea, lemonade and water urged by mouth. Enemata not well retained by this patient, but when given by Murphy drip the glucose solution was well borne. Recovery in three or four days.

TABLE III.—*Case III* (*Br—*) : *Urine Analysis.*

Total acetone in blood ·145 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Jan. 22	1250	10·4	1·3	7·5	3·7	—
,, 23	1130	6·1	1·0	4·0	0·1	—
,, 24	860	3·8	0·5	2·5	—	Carbohydrate meals.
,, 25	965	5·7	0·8	3·7	—	—

Case IV (*NN—*).*

Primipara, aged 19; moderately severe case. Previous history uneventful. Physical findings negative, except for slight icterus. Treatment: Nutrient enemata, 10 per cent. glucose in saline, 6 oz. *t.i.d.* and fluids urged by mouth. Previous to admission patient had been unable to keep down even water, but was easily able to take small amounts of fluid in hospital, and soon augmented her water intake. Clinical condition rapidly improved and patient was eating on the fourth day. After clinical history uneventful. This case is exceptional in that the acetone bodies continued in the urine for a long time, even when the patient was taking large amounts of food, although there was no accompanying clinical manifestation.

TABLE IV.—*Case IV* (*NN—*) : *Urine Analysis.*

Total acetone in blood ·172 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Feb. 9	500	7·3	1·7	4·4	6·6	—
,, 10	515	7·0	1·9	3·8	6·9	—
,, 11	525	4·7	1·3	2·5	4·5	—
,, 12	840	4·3	1·3	2·0	4·3	Carbohydrate meals.
,, 13	1250	5·1	1·6	2·3	2·5	,,
,, 14	550	2·9	·8	1·1	1·1	,,
,, 15	600	2·3	·6	1·0	1·4	,,
,, 16	860	2·2	·5	1·0	1·3	,,
,, 17	810	3·2	·7	1·7	1·0	Protein in diet.
,, 18	580	1·8	·4	·8	·4	,,
,, 19	970	3·0	·5	1·3	·5	,,
,, 20	980	2·5	·4	1·3	·5	,,
,, 21	500	1·9	·2	1·2	·2	,,
,, 22	970	3·4	·5	2·0	·7	,,
,, 23	620	2·0	·3	1·2	·4	,,
,, 24	820	4·1	·4	2·4	·8	,,
,, 25	1210	4·3	·2	2·9	·5	,,
,, 26	400	1·5	·2	·9	·1	,,
,, 27	620	2·3	·2	1·3	·2	,,

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* A full report of this case is to be found in a previous paper (Harding and Watson, 1922).

Case V (C—k).

Primipara, aged 32; about three months pregnant. Had been vomiting most of the pregnancy. Of a distinctly nervous type, and gave a history of biliousness at the menstrual periods. No food for the two days previous to entry into hospital. Physical examination normal. Signs of dehydration, however, were present. Treatment: Nutrient enemata of 10 per cent. glucose in saline, with sodium bromide gr. xl, ʒ oz. *t.i.d.* This case for the first two or three days did not progress favourably, but the continuance of the enemata and the fluids by mouth produced a sudden change, both clinically and metabolically. This is the only case which showed any evidence of true acidosis, the CO_2 content of the alveolar air being found to be 3·1 per cent. Casts were also found in this urine on two days. After the sudden change on November 9th the patient's condition rapidly improved, although she was subject to occasional mild attacks of nausea.

TABLE V.—*Case V (C—k) : Urine Analysis.*

Total acetone in blood ·115 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Nov. 6	555	6·9	·9	5·0	6·4	—
„ 7	1105	8·0	1·5	5·7	8·0	—
„ 8	1510	6·8	1·6	4·4	8·2	—
„ 9	755	4·2	·7	2·4	·8	Carbohydrate meals. Enemata discontinued.
„ 10	1125	3·2	·4	1·9	—	Protein in diet.
„ 11	1750	4·8	·4	3·4	—	„

Case VI (M—).

Multipara, aged 35, about 2½ months pregnant. Vomiting for two months previously. Previous two pregnancies had been marked by nausea, but not by excessive vomiting such as this. Patient was a large, florid, well-nourished woman. Slight cough, but chest examination gave negative findings. Tongue and skin moist, lips were cracked, and the patient felt thirsty. Conjunctivæ

TABLE VI.—*Case VI (M—) : Urine Analysis.*

Total acetone in blood ·201 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Feb. 21	370	5·3	1·0	3·4	2·0	—
„ 22	450	4·5	·7	3·2	·7	Interstitial glucose.
„ 23	1080	7·6	·8	5·8	·2	Carbohydrate meals.
„ 24	1260	4·2	·5	2·9	·1	„
„ 25	2260	5·9	·5	4·1	—	„
„ 26	2130	3·9	·4	2·6	—	Protein in diet.

were bile-stained; epigastric pain on pressure. Treatment: Nutrient enemata of 10 per cent. glucose in saline, bromide gr. xx, 6 oz. *t.i.d.* This produced a little improvement, but not marked, and on the third day 500 c.c. 10 per cent. sterile glucose in saline were given interstitially. The patient vomited once during the night, and once the day following, but afterwards was free from symptoms. Six days after admission she was able to take low-protein, high-carbohydrate diet. After-history uneventful.

*Case VII (B—k).**

Multipara, aged 27. Previous pregnancy terminated by Cæsarian section at eight months after continuous vomiting. Living child had been obtained. Patient was extremely thin and ill nourished. No jaundice or epigastric pain; vomiting frequent and no food taken for previous week. Physical examination showed a pelvis so contracted that the only possibility of obtaining a living child lay through another Cæsarian section, otherwise negative. Treatment: 10 per cent. glucose in saline 6 oz. *t.i.d.*, with bromide gr. xx and chloral gr. x in evening enema. Fluids urged by mouth. Patient did not improve under this treatment, and on the third day 300 c.c. 10 per cent. glucose in saline were given interstitially. Temporary improvement seemed to follow this, but there was again retrogression, and two days later 1000 c.c. 5 per cent. sterile glucose in saline were given intravenously. This, again, seemed to effect a little improvement, but the change was not lasting. Therapeutic abortion was carried out on February 1st, and the day following the nutrient enemata were recommenced. In two days the patient was well, able to eat small meals, and the acetone bodies had disappeared from the urine.

TABLE VII.—*Case VII (B—k): Urine Analysis.*

Total acetone in blood ·417 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Jan. 25	400	5·8	·6	4·5	4·7	—
,, 26	570	10·5	1·8	6·9	6·7	—
,, 27	450	7·4	1·4	5·1	5·0	—
,, 28	375	5·9	1·2	3·8	4·3	300 c.c. 10 per cent. glucose interstitially.
,, 29	565	8·3	2·0	4·9	8·0	—
,, 30	260	4·4	·8	2·8	2·0	1000 c.c. 5 per cent. glucose intravenous.
,, 31	525	6·2	·8	4·6	1·5	—
Feb. 1		Abortion.				—
,, 2	1000	7·5	1·2	5·0	2·5	—
,, 3	670	2·3	·2	1·8	—	Carbohydrate meals.

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* A full report of this case is to be found in a previous paper (Harding and Watson, 1922).

EXCRETION OF ACETONE BODIES.

The theory which has been put forward in previous papers postulated a carbohydrate disturbance occurring in the maternal liver as the primary factor concerned in the ætiology of the nausea and vomiting of pregnancy with consequent production of acetone bodies in the blood and urine. It has been postulated by Shaffer (1921[1], 1921[2], 1922) and others that the production of acetone bodies is dependent, upon a balance between the ketogenic and antiketogenic substances undergoing catabolism. These substances include carbohydrate, protein and fat molecules, but by far the preponderance of antiketogenic molecules is derived from carbohydrate. Whether or no the "acetone bodies" give rise to a disturbance of acid-base equilibrium is a matter which will be discussed later in this paper, and until such a disturbance is shown, it would be better to use the term *ketosis* in this connection. For the present, we wish to discuss their relationship to the clinical findings in the series of patients whose histories we have just, in brief, described. Of the seven cases, six improved rapidly under the treatment, and by the fifth or sixth day were free from all vomiting, and were eating three small meals a day with relish. In all except Case 4 the acetonuria had ceased in a like length of time. These results are typical of all the cases we have seen in which the use of glucose and water, together with rest and isolation, have been successful in overcoming the nausea and vomiting of pregnancy. In Case 4 the acetonuria persisted in a very mild degree for several days after clinical recovery had become an established fact, though previous to this there had been a rapid decline in the total acetone excretion coincident with a rapid improvement in the clinical condition. We have only met with one other case similar to Case 4, in which a mild acetonuria persisted long after the patient was free from symptoms. Case 7 represents the only case upon which we have quantitative data where the treatment failed to produce an improvement in the clinical condition. In spite of the fact that glucose was given both by the interstitial and intravenous route, as well as by rectum, the patient was vomiting as freely at the end of seven days as at the beginning. Here the acetone bodies fluctuate markedly in contrast to the other cases. On the sixth and seventh day it would seem as though they were going to disappear from the urine; the third and fourth days, however, had been marked with a progressive fall in their excretion, only to be followed on the fifth day by a rise to a greater height. Thus, an extremely vigorous administration of glucose in this case failed to abolish, or maintain at a permanently low level, the excretion of the acetone bodies. In this way it is in contrast with the other cases.

CONCENTRATION OF ACETONE BODIES IN THE BLOOD AND URINE.

Considerable interest attaches to the concentration of the acetone bodies in the blood and the rate of their excretion into the urine, as the balance between the rate of production and the rate of elimination of these acids will be a powerful factor in determining the presence or absence of an acidosis.

Reference to the tables already given shows that the concentrations of "acetone" in the blood are not remarkably high. In seven cases, only one showed a concentration as high as 0·4 gm. per litre, while all the others varied from 0·1 to 0·2 gm. per litre. Comparisons with the "acetone" concentration in the blood in other conditions are difficult, but our figures are not to be compared with those for blood in diabetes associated with a depletion of the bicarbonate reserve of the plasma, and with the clinical manifestations of acidosis. In discussing the general results obtained on the blood "acetones" in diabetes, Van Slyke and Fitz (1917) remark that the well-managed case will show from 0·2 to 0·4 gm. per litre. Such a case of diabetes would be one in which the production of the acetone bodies had been kept sufficiently well under control so as to eliminate all immediate danger of coma, and the blood would show a normal or only slightly lowered alkaline reserve. This agrees with the findings in nausea and vomiting of pregnancy. In our own series of cases we looked for evidence of acidosis in the CO_2 content of alveolar air, using the Fridericia apparatus. With one exception (Case V) we obtained normal figures, and even in this case the value had risen to normal two days subsequent.* Losee and Van Slyke (1917) have examined the plasma bicarbonate reserve in pernicious vomiting and found normal or slightly subnormal values. There is, then, no evidence in the blood or the alveolar air that anything but the mildest disturbance is taking place in the direction of acidosis. Incidentally, one may note here that the giving of bicarbonate in vomiting of pregnancy has no justification from this standpoint.

Contrasted with the concentration in the blood, the concentration of the "acetone" in the urine is sometimes remarkably high. The daily excretion, as seen in the tables attached to the individual cases, may also be quite large in amount. The acids, then, are eliminated from the body in high concentration and large amount. Indeed, we may remark that the concentration of the total acetone bodies in three of our cases (IV, V and VII) is higher than the maximum given for diabetes. Allen, Stillman and Fitz (1919), in their studies on diabetes, came to the conclusion that the possible concentration of the acetone bodies in the urine was limited, the highest observed being between 9 and 10 gm. per litre. Whilst we would not like to insist that the maximum concentration given by Allen, Stillman and Fitz might not be exceeded in diabetes, still, it does demonstrate clearly that the concentration of the acetone bodies in vomiting of pregnancy may be extraordinarily high.

The total amounts of "acetone" excreted, too, are large, comparable in amount with those found in many cases of severe diabetes. They are distinctly higher than figures for the usual "acetone" excretion in starvation, though there are notable exceptions.

In conclusion, then, there is in vomiting of pregnancy a large production of aceto-acetic and β-oxybutyric acids, which is rapidly and promptly eliminated in the urine in high concentration, leaving the concentration in the blood at low levels. They do not accumulate in the blood to the extent found in diabetic comas, and their presence is not associated with any disturbance of the alkaline reserves. It is pertinent, therefore, to inquire into the manner in which they are eliminated, especially to see if the high ammonia coefficients observed in this condition are related to their excretion as is the case in starvation.

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* The results by the Fridericia apparatus are sometimes low without a corresponding low plasma bicarbonate (Stillman, Van Slyke, Cullen and Fitz, 1917).

EXCRETION OF AMMONIA.

A great deal of attention has been paid to the excretion of ammonia in the vomiting of pregnancy, unfortunately without, at the same time, paying attention to the excretion of acid. The misleading employment of a figure known as the "ammonia coefficient," together with its only occasional estimation instead of the determination of the ammonia day by day, has led to many erroneous conclusions. We do not wish to say that a number known as the "ammonia coefficient" or the "ammonia index" may not be used if it is so desired, but its interpretation is not so simple as that of the actual number of grammes of ammonia excreted day by day.

In our cases it is obvious (Tables I–VII) that the ammonia excretion follows in a general way the excretion of acetone bodies, and that diminution or increase in urinary ammonia is usually accompanied by similar changes in the acetone excretion, especially in cases where large amounts of "acetone" are being eliminated. That complete harmony between the curves of the two bodies does not exist is not to be wondered at. Ammonia is only one of the bases which play a part in the maintenance of the acid-base equilibrium of the body, and is normally present in the urine without the occurrence of "acetone." Gilliatt and Kennaway also observed the high ammonia index to be accompanied by an increased excretion of acetone bodies, though in neither of their cases do they possess complete data on this latter excretion. In urine containing smaller amounts of "acetone," the correspondence between the two excretions would necessarily not be so complete, for, as we have just stated, normal urine always contains a certain quota of ammonia without the presence of acetone bodies. Such results are similar to those indicated in starvation in the earlier classical researches of Brugsch (1905) and of Cathcart (1907).

The publications of Henderson and Palmer (1913) and of Fitz and Van Slyke (1917) have shown that the body makes every effort it can to avoid any wastage of fixed base. It does so in two ways:

(1) By the production of ammonia at the expense of urea.
(2) By increasing the acidity of the urine.

That this first mechanism for preserving fixed base is called into play in the vomiting of pregnancy we have just shown. Unfortunately we did not determine the magnitude of the possible second factor. It is, however, well known that urines in vomiting of pregnancy are highly acid, and Gilliatt and Kennaway in their Cases I and II show the high acidity with high acetone excretion, and a much lowered acidity when the urine approaches its more normal composition, though most of their figures are percentages and not daily amounts. Consequently we readily conclude that in pernicious vomiting the body endeavours to get rid of aceto-acetic acid and β-oxybutyric acid by eliminating part of them as such in the urine. In our opinion, then, these abnormal acids are eliminated from the body by means of the two ordinary mechanisms just stated. There is thus no reason to suppose in the average that any depletion of fixed base should occur from the body's store of reserves, though doubtless occasional cases will arise in which the production of β-oxybutyric and aceto-acetic acids may be so intense that these two are drawn upon to preserve the acid-base equilibrium, and a certain amount becomes lost to the body. We do not wish at this stage of our inquiry to discuss the mechanism of the transport of "acetone" in the blood, though in view of what we have just stated the papers of Nash and Benedict (1922), and Gamble, Ross and Tisdall (1922) form a very valuable guide.

EXCRETION OF UREA.

The earlier investigators who were impressed by the finding of a fatty degenerated liver at autopsy in cases of pernicious vomiting attempted to find metabolic evidence of this disturbance. They pointed to the raised ammonia coefficients and the lowered urea nitrogen percentages as evidence of serious hepatic lesions. The lowered urea nitrogen percentage found by Ewing and Wolf (1907) was confirmed by Losee and Van Slyke (1917). On the whole, our series (see Tables I–VII) does not show such raised ammonia indexes and such lowered urea nitrogen percentages as may be found in the work of other investigators. Our lowest figures for urea nitrogen percentages are to be found in Case IV, in which on one day the urea nitrogen excretion represented only 43·8 per cent. of the total nitrogen. In discussing these low figures it must be borne in mind that in this patient extraordinarily low amounts of total nitrogen were observed in the urine, and as the urea fraction represents the major variable fraction of the total nitrogen, any variation in this latter is at once reflected in an enhanced manner in the urea nitrogen percentage. In order, however, to show that these low values for urea are due to the conversion of either part of the urea itself, or one of the precursors of urea, we added together the urea nitrogen percentage and the ammonia index. The sum of the two in all cases gave a value which is comparable with the value found in normal individuals when excreting similar amounts of total nitrogen. The values ranged from 80 per cent. to 95 per cent. except in Case IV, where, with the extraordinarily low total nitrogen excretion, the sum total of the urea nitrogen percentage and the ammonia index was correspondingly lowered.

EXCRETION OF TOTAL NITROGEN.

The nitrogen balance in pregnancy has been a matter of considerable interest and importance. Our exact knowledge commences with the work of Bar, and from his work and the investigations of Murlin and Gammeltoft the conclusion has developed that in normal pregnancy there are at least two stages of protein metabolism (*vide* Murlin, 1917). The first occurring, in the early part of pregnancy, shows either a negative nitrogen balance, or a lowered positive balance when compared with the balance of the previous non-pregnant period; and a second stage in later pregnancy always characterised by a positive nitrogen balance. These conclusions have been developed from work on animals, mainly dogs and rabbits, but are borne out in some degree by the observations of Landsberg and Wilson on women, although actually a negative nitrogen balance in women has never been seen in early pregnancy. As the period of negative nitrogen balance in the dog corresponded closely with the development of the placenta, and as at about the point of the completion of that organ the negative balance changed to a positive one, and as in one of his animals Bar observed a period of distaste for food and actual vomiting, he was led to the conclusion that associated with the nitrogen loss was the so-called "physiological" vomiting of pregnancy. In this conclusion he was supported by Murlin and to a certain extent by Gammeltoft, and in this connection it is of interest to note that the cases of early pregnancy studied both by Landsberg and by Wilson had shown no trace of nausea or vomiting. Indications of the existence of this negative nitrogen balance in early pregnancy had been found by observers earlier than Bar, and though their experiments were not decisive, they had been led to the conclusion that this nitrogen loss could not be prevented by feeding, in which they were later supported by Gammeltoft. The cause of this destruction of protein was very clearly defined by Murlin as due to the parasitic life of the freshly-growing ovum, which, attacking the uterine wall by means of a series of proteolytic enzymes, derived its energy and material for growth exclusively from protein. The maternal organism, at this point, was concerned solely with obtaining a means of defence against the growing invader, and the placenta was devised as an organ of control, interposed between the fœtus and the mother. At this point the relation of parasite and host changed to one of symbiosis.

In Tables I–VII we have given the total nitrogen excretion of our series of cases. It is evident at once from an inspection of the tables that under the influence of the treatment the total nitrogen excretion shows a downward tendency. Were we dealing with a simple case of starvation such a result would be at once expected, as the sparing action of carbohydrate upon protein is a well-acknowledged fact. That, however, small amounts of carbohydrate such as we administered in these cases (50 gm. to 100 gm.) should have so marked an effect upon the total nitrogen excretion is somewhat a matter of surprise. Are we to connect nausea and vomiting of pregnancy with protein destruction brought about solely by the parasitic action of the fœtus? This lack of harmony between the course of the total nitrogen excretion in our cases and the theory of "physiological" vomiting of Bar becomes even more apparent when we notice the almost complete correspondence between the excretion of total nitrogen and the excretion of ammonia. We have already designated the part played by ammonia and its relationship to the excretion of "acetone," and it would thus appear much more likely that the total nitrogen excretion, and consequently the extent of the tissue destruction which takes place in these cases, is governed by the same factor that controls the excretion of "acetone" and ammonia. The results which we have just given are borne out in part by the figures of Underhill and Rand (1910) in their Cases 1, 2 and 4. It would thus appear possible that lack of carbohydrate is responsible for the perverted fat catabolism and the tissue destruction which we see in vomiting of pregnancy, and that protein destruction takes place to provide its moiety of glucose molecules.

The work thus described would give a dominating influence to carbohydrate in vomiting of pregnancy, but again one must compare the conclusions with those derived from a study of starvation. Landergren (1903), from his studies of nitrogen metabolism in starvation, as early as 1903 expressed the belief of the "absolute value of carbohydrate." These conclusions were supported by Cathcart (1909–10), but seem to have been overlooked or have lapsed into oblivion in later years. Similar conclusions can thus be drawn from a study of pernicious vomiting and starvation. The fundamental lack in both is carbohydrate.

CONCLUSIONS.

a. The excretion of "acetone" in the majority of cases of nausea and vomiting of pregnancy parallels the clinical condition.

b. The effect of abortion is to diminish the production of "acetone."

c. Tissue destruction in nausea and vomiting of pregnancy when under the influence of small amounts of carbohydrate is governed by the demand for further supplies of carbohydrate.

d. The results are similar to those observed in some cases of starvation, but in pregnancy the demand for carbohydrate, and consequently the disturbance following its lack, is more intense than in starvation.

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REFERENCES.

Allen, F. M., Stillman, E., and Fitz, R.—(1919) *Monographs of Rockefeller Inst. for Med. Research*, No. 11, p. 107.
Brugsch, T.—(1905) *Ztsch. f. exper. Path. u. Ther.*, **1**, 419.
Cathcart, E. P.—(1907) *Biochem. Ztsch.*, **6**, 109.—(1909–10) *J. Physiol.*, **39**, 311.
Duncan, J. W., and Harding, V. J.—(1918) *J. Canad. Med. Assoc.*, **7**, 1057.
Ewing, J., and Wolf, C. G. L.—(1907) *Amer. J. Obstet.*, **55**, 289.
Fitz, R., and Van Slyke, D. D.—(1917) *J. Biol. Chem.*, **30**, 389.
Gamble, J. L., Ross, S. G., and Tisdall, F. F.—(1922) 34th Annual Meeting of the American Pediatric Society, Washington, D.C.
Gilliatt, W., and Kennaway, E. L.—(1918–19) *Quart. J. Med.*, **12**, 61.
Harding, V. J.—(1921) *Lancet*, **2**, 327.
Harding, V. J., and Watson, B. P.—(1922) *Ibid.*, **2**, 649.
Henderson, L. J., and Palmer, W. W.—(1913) *J. Biol. Chem.*, **14**, 81.
Landergren, E.—(1903) *Skand. Arch. f. Physiol.*, **14**, 112.
Losee, J. R., and Van Slyke, D. D.—(1917) *Amer. J. Med. Sci.*, **153**, 94.
Murlin, J. R.—(1917) *Amer. J.Obstet.*, **75**, 922 (Discussion of the Nitrogen Balance in Pregnancy).
Nash, T. P., and Benedict, S. R.—(1922) *J. Biol. Chem.*, **48**, 463.
Shaffer, P. A.—(1921)[1] *Ibid.*, **47**, 433, 449.—(1921)[2] *Ibid.*, **49**, 143.—(1922) *Ibid.*, **54**, 399.
Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R.—(1917) *Ibid.*, **30**, 407.
Underhill, P. F., and Rand, R. F.—(1910) *Arch. Int. Med.*, **5**, 61.
Van Slyke, D. D.—(1917) *J. Biol. Chem.*, **32**, 455.
Van Slyke, D. D., and Cullen, G. E.—(1914) *Ibid.*, **19**, 211.
Van Slyke, D. D., and Fitz, R.—(1917) *Ibid.*, **32**, 495.

THE RELATIVE CONCENTRATION RATIOS OF SOME CONSTITUENTS OF THE URINE.

II.—THE RELATIVE CONCENTRATION RATIOS OF UREA, CREATININE, INORGANIC PHOSPHATE AND URIC ACID.

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SEVERAL observations have been made that the various "no-threshold" bodies may be increased unequally in the urine under certain conditions, but the results, as far as concerns theories of kidney function, are often not quite conclusive, owing to the absence of definite information as to the possible variations in their amounts in the blood at the same time.

Marshall (1920) has shown that in water diuresis, although the volume of urine is largely increased, yet the output of creatinine is unchanged, while that of urea is only slightly raised.

Carr (1921) has reported similar results. The output of urea and sulphates is always increased, that of NH_4 and P_2O_5 is generally, while the output of creatinine shows no measurable alteration. The variations in the outputs of urea and sulphates are not parallel. The volume of urine always increases to a still greater extent, so that there is in all instances a decrease in the percentage amounts present.

In the diuresis following increased renal blood-flow from section of the renal nerves, Marshall and Kolls (1919) and Marshall and Crane (1921) have obtained similar results; the creatinine excretion was unaffected, the output of urea, SO_4 and P_2O_5 somewhat raised, while the volume of urine was largely increased.

Marshall's experiments on water diuresis in man were controlled by comparing the results with those obtained at the same hours of the day, and under the same conditions, except that no water was drunk. He states that the results cannot be due to plasma changes, since even after copious water drinking dilution of the blood is extremely small (Haldane and Priestley, 1916; Priestley, 1921); moreover, analyses of the plasma in dogs showed no significant changes in urea and creatinine, although chlorides fell, accompanying an increased excretion in the urine. In these experiments some time (up to 2 hours) elapsed between the drawing of the two blood samples.

In one experiment the concentration ratios of urea and creatinine (from estimations on one hour's urine and one blood sample) were different, the latter being concentrated about one and a half times more than the urea.

The results suggest that, in diuresis, assuming the amounts in the blood to remain fairly constant, the concentration ratios of creatinine, urea and phosphates should *fall*, this fall being greatest in the case of creatinine, and less marked in the case of urea and phosphates. The concentration ratio of creatinine relative to that of urea would be less, while the relationship of the ratios of phosphate and urea might be unaltered, or the former might show a diminution relative to the latter.

While this work was in progress a paper was published by Mayrs (1922); using rabbits, he injected various no-threshold bodies in pairs, and after about five minutes, when a sufficiency of urine had been collected, bled the animals to the extent of three-quarters of their blood volume, and performed the estimations on the plasma and urine. He found that SO_4, P_2O_5 and creatinine were concentrated to about the same extent and 1·5 to 2·75 times more than the urea. He suggested that the urea was reabsorbed in the tubules, possibly subserving some useful function in the organism. This conclusion was confirmed by experiments in which one kidney secreted against pressure, and in these the effect of resistance on the output of urea was greater than on that of SO_4.

Finally, it might be mentioned that there are a number of observations obtained in cases of renal disease, and from the use of drugs, which suggest that the function of the kidneys towards any one of the no-threshold bodies may be altered without a corresponding alteration in their relationship to the others, but the relative concentration ratios have not been accurately determined.

METHODS.

In this series of experiments an attempt has been made to compare the concentration in blood and urine simultaneously, of urea, creatinine and inorganic phosphate, and in a few, of uric acid in addition.

The general plan was to inject a solution of the substances about to be examined, and after a short time take a blood sample, and a further sample after a variable length of time, during which the urine was collected.

The reasons for injecting a solution of the substances whose concentration ratios were to be determined were three in number; first, a greater amount in the blood enabled smaller samples to be collected, thus obviating too great a loss of blood, while the error incidental to calculating the concentration ratio from the mean of two samples was diminished; secondly, the greater amounts present per cent. in the urine, together with the resultant diuresis, if the amounts injected were large enough, shortened the time of collection of the urine—a smaller volume being necessary for the requisite estimations. In addition, this shorter time allowed less for material alterations to occur in the quantities present per cent. in the plasma.

Finally, with higher plasma percentages, extraneous factors have less influence on the excretion rates of the substances studied (Addis and Watanabe, 1917).

The animals used have been cats: under ether, followed in later experiments by C.-E., cannulæ were inserted into a carotid, for recording blood-pressure, and into the trachea. Preparations were also made for injecting the solution into a femoral vein, and for withdrawing blood, usually from the opposite carotid artery. A catheter was inserted into the bladder by making an incision in the urethra just above the symphysis pubis. In all experiments except the first (where urethane was given) the animal was then decerebrated by trephining. No further steps in the experiment were taken for at least half an hour.

The solution was then injected, a blood sample obtained, and the collection of urine begun. When sufficient urine had been obtained a second blood sample was drawn. Sometimes the experiment was continued, further samples of blood and urine being obtained. The bladder was either emptied by pressure, or the body was ligatured, or clamped off from the base, so that the urine flowed directly into the catheter. The blood was drawn into paraffined tubes containing sodium oxalate (powdered) in the proportion of 20 mg. to 10 c.c. of blood, and centrifuged; the plasma was used for the analyses.

The analytical methods used were the same as previously described (Underhill, 1923).

RESULTS.

The results are set out in Tables I, II and III. The amounts and natures of the substances injected are given in the second column followed by the time which elapsed before the first blood sample was drawn. In the two experiments in which uric acid was injected, it was found that the alkalinity of the Na_2HPO_4 was not great enough to dissolve it completely without the addition of a little NaOH.

During the following period urine was collected, and the total volume obtained and the times of collection are given in the next columns. Then follow the results of the analyses of the plasma and urine; in the case of the former the results of the two analyses are given, each pair of figures thus representing the amounts present in the plasma at the beginning and end of the period during which the urine was collected.

The amount in the plasma for calculating the concentration ratio is taken as the mean of the two analyses. In the last column, or columns, the ratio is compared with that of urea, taking the urea concentration ratio as = 1.

In the case of the first two experiments the analyses were performed on whole blood instead of plasma, while in the first the animal was under urethane and not decerebrated.

It will be noticed on examination of the Tables that there is a general tendency for the concentration ratio to vary in the course of a single experiment, in the same direction as the concentration of the substance in question in the *urine*. This is explicable on the assumption that the concentration in the urine depends in part on the rate at which the urine is secreted: a more rapid secretion allows less time for water to be reabsorbed in the tubules, and the concentration in the urine falls and with it the concentration ratio. On the other hand, an inverse relationship between the

TABLE 1. (PART 2)

EXPERIMENT Nº	INJECTION	PERIOD BETWEEN INJECTION & FIRST BLOOD SAMPLE IN MINS.	VOL OF URINE IN C.C	TIME OF COLLECTION OF URINE IN MINS.	UREA			CREATININE			C/Uv
					PLASMA* mg PER 100c.c	URINE mg PER 100 c.c	CONCENTRATION = Uv	PLASMA* mg PER 100c.c	URINE mg PER 100c.c	CONCENTRATION = C	
1† (MALE) 3·5 K	UREA 0·12g CREATININE 0·005g IN 5 C C SALINE	44	5·0	51	51·6 55·2	726	13·6	1·3 1·3	4·3	33	2·4
2 (MALE) 3·2 K	(a) UREA 0·05 g CREATININE 0·005g IN 25 C C SALINE	7	5·7	39	43·1 41·7	964	22·7	1·4 1·3	59	43·7	1·9
	(b) UREA 0·02g CREATININE 0·002g IN 10 C C SALINE	2	7·9	42	50·4 61·9	412	7·3	1·5 1·4	29·5	20·3	2·8
8 (MALE) 3·5 K	UREA 0·25g CREATININE 0·01g IN 5 C.C. SALINE 50 C C SALINE	49 5	1·95	43	72·0 71·3	2775	38·7	1·7 1·6	254	154	4·0
9 (MALE) 2·75 K	UREA 1·0g CREATININE 0·05g IN 50 C C SALINE	2	3·6	26	157·0 105·0	3644	27·8	8·2 3·0	470	84·0	3·0
11 (MALE) 4·5 K	UREA 1·0g CREATININE 0·05g IN 50 C C SALINE	2	(a.) 1·9	15	141·0 95·0	947	8·0	6·1 3·7	90	18·4	2·3
			(b) 1·6	20	95·0 104·0	1193	12·0	3·7 3·4	160	45·0	3·7

† CAT UNDER URETHANE

* IN EXPERIMENTS Nº 1 & Nº 2 THE ANALYSES WERE PERFORMED ON WHOLE BLOOD

TABLE 2. (PART 2)

EXPERIMENT Nº	INJECTION	PERIOD BETWEEN INJECTION & FIRST BLOOD SAMPLE IN MINS.	VOL OF URINE IN C.C.	TIME OF COLLECTION OF URINE IN MINS	UREA			CREATININE			INORGANIC PHOSPHORUS			URIC ACID			C/Uv	P/Uv	UA/Uv
					PLASMA: mg PER 100 c.c	URINE. mg PER 100 c.c.	CONCENTRATION = Uv	PLASMA: mg PER 100 c.c	URINE: mg PER 100 cc.	CONCENTRATION = C	PLASMA: mg PER 100 c.c.	URINE: mg PER 100 c.c.	CONCENTRATION = P	PLASMA: mg PER 100 c.c	URINE: mg PER 100 c.c	CONCENTRATION = UA			
12 (MALE) 3·64 K	UREA 1·0g CREATININE 0·05g $Na_2HPO_4,12H_2O$, 0·5g URIC ACID 0·025g IN 12·5 c.c DISTILLED WATER 50 c.c SALINE	10 7	3·15	20	117 101	2400	22	3·9 3·2	195	55	12·6 8·3	253	24	0·65 0·53	50·6	[illegible]	2·5	1·1	3·9
13 (MALE) 4·0 K	UREA 1·0g CREATININE 0·05g Na_2HPO_4 $12H_2O$ 0·5g URIC ACID 0·25g IN 27·5 c.c. DISTILLED WATER	34	(a) 2·8	10	73·7 92·5	989	12	1·9 2·4	92	43	7·7 11·5	109	11·0	3·6 3·0	122	37	3·6	0·95	3·1
			(b) 4·45	13	92·5 101·0	728	7·5	2·4 2·4	63	26	11·5 8·9	90	9·0	3·0 1·7	69	29	3·5	1·2	3·9
			(c) 4·62	15	101·0 103·2	759	7·4	2·4 2·4	58·5	24	8·9 8·5	72	8·0	1·7 1·1	50	36	3·3	1·1	4·8

concentration in the urine and the concentration ratio may occur: in these instances an explanation must be sought in the amounts available for excretion, as indicated by the behaviour of the concentration in the plasma.

The creatinine is always concentrated to a greater extent than the urea—from two to five times more in the different experiments. From experiments 11 and 13 it will be seen that the relative concentration ratios follow the movements of the urinary concentrations of urea and creatinine. This can be explained on the basis of the filtration reabsorption theory; as the passage of the urine along the tubules becomes slower, so there is more time for water to be absorbed and with it some of the urea, if this modification of the theory be accepted; and the reverse occurs when the concentrations in the urine fall.

TABLE 3. *(PART 2)*

EXPERIMENT Nº	INJECTION	PERIOD BETWEEN INJECTION & FIRST BLOOD SAMPLE IN MINS.	VOL. OF URINE IN C.C.	TIME OF COLLECTION OF URINE IN MINS.	UREA			CREATININE			INORGANIC PHOSPHORUS			$\frac{C}{Uv}$	$\frac{P}{Uv}$
					PLASMA mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = Uv	PLASMA mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = C	PLASMA mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = P		
16 (MALE) 4·1K	UREA 1·0g CREATININE 0·1g $Na H_2 Po_4$, H_2O, 1·0g IN 20 C.C. DISTILLED WATER	10	(a) 3·3	30	120 103	842	7·5	9·7 5·7	260	33·8	33·7 22·6	409	14·5	4·5	1·9
			(b) 2·6	15	103 106	936	9·0	5·7 4·8	242	46·1	22·6 18·0	456	22·5	5·1	2·5
			(c) 1·65	30	106 117	990	8·9	4·8 4·9	223	46·0	18·0 17·9	438	24·4	5·2	2·7
17 (FEMALE) 2·75 K	UREA 1·0g CREATININE 0·1g $Na_2 H Po_4$, $12H_2O$, 2·5g IN 25 C.C. DISTILLED WATER	8	(a) 5·8	10	125 95	768	7·0	11·9 7·3	140	14·6	49·3 35·2	466	11·0	2·1	1·6
			(b) 3·9	12	95 105	763	7·6	7·3 6·2	146	21·6	35·2 28·0	486	15·4	2·8	2·0
			(c) 4·4	10	105 97	849	8·4	6·2 5·3	126	21·9	28·0 25·4	452	16·9	2·6	2·0

In experiments 16 and 17 the concentrations of urea and creatinine in the urine vary in opposite directions, with a tendency for the relative concentration ratio to follow the urea concentration: variations from this relationship possibly depend on the fact that the plasma concentrations of the two substances do not always vary in the same direction.

In experiment 2, while there is a fall in the urinary concentrations, yet there is a rise in the relative concentration ratio, and this is accompanied by a rise in the concentration of urea in the blood. It is possible that the fall in urinary concentrations indicates a lack of appreciable excess to be excreted rather than a diuresis; only very small quantities had been injected previously. These results, then, agree in most instances with the conclusions deduced from the experiments of Marshall and Kolls (1919) and Marshall and Crane (1921).

The general results of my experiments agree, in spite of the fact that the plasma concentrations may vary in the same or in opposite directions, or remain almost constant. Under the conditions of the experiment it is considered that where a substance shows an almost constant plasma concentration as estimated from the two samples, there has been no appreciable fluctuation of the percentage amount in the plasma during the time of collection of the urine, and that the concentration ratio represents fairly accurately the relation between the plasma and urinary concentrations. The only other source of error to be considered is the possibility that the urine collected may not accurately represent the urine secreted during the time between the drawing of the two samples. The urine contained in the catheter was always allowed for, and the bladder was always emptied, so that only the urine in the tubules of the kidney and ureter need be considered: in the cat's kidneys this possibly amounts to about 0·2 c.c. (calculated from figures given by Cushny, 1917). However, this error is presumably not great, considering the similarity of the results obtained with different rates of secretion of urine; in cases where a diuresis occurred the error becomes negligible.

The fact that the results obtained when the plasma concentrations are constant are comparable with those obtained when these concentrations varied, suggests that taking the mean plasma concentration for calculating the concentration ratio introduces no appreciable error, the concentration in the plasma falling or rising, more or less rapidly, from the first to the second figure, without any intermediate considerable fluctuations.

In those experiments in which the plasma concentration remained almost constant, the excretion of substances in the urine must be balanced by a corresponding absorption from the tissues, or by a concentration of the blood. Attempts have been made to follow the latter by means of hæmoglobin estimations. After the dilution following the injection the concentration remains almost constant, usually with a tendency to increase during the course of the experiment.

Turning now to the results reached with inorganic phosphate and uric acid, it is not wished to lay great stress on those obtained with the latter, except to point out that the degree of concentration compared with that of urea is of the order of that of the creatinine. Since, in the cat, uric acid is not an end-product of metabolism, but is further converted into allantoin (Jones, 1920), its disappearance from the blood is much more rapid than in the case of the other substances investigated, giving a false view of the amount to which it is concentrated in the urine relative to its plasma percentage. In experiment 13 (*a*), however, the plasma concentration only fell from 3·6 to 3·0 mg. per 100 c.c. in ten minutes, and in that period its concentration ratio relative to that of urea was 3·1, while that of creatinine was 3·6. The results, as far as they go, do not suggest that uric acid is other than a no-threshold substance in the cat.

When the disodium phosphate was injected in experiments 12 and 13, the results showed a concentration ratio for inorganic phosphorus of about the same value as that of urea; further experiments, however, gave a figure which was intermediate between that of urea and creatinine. In experiment 16 the acid sodium phosphate was used in much greater amount than the alkaline in Nos. 12 and 13—the content in phosphorus being about five times greater. It was possible that the difference in the results was due to the use of the acid instead of the alkaline phosphate, since Nagayama (1920) found the urea-excreting power of the kidneys in rabbits less after the administration of the acid phosphate, as compared with a neutral phosphate mixture of the same phosphorus content, both being accompanied by the same amount of urea. However, experiment 17, in which the phosphorus content of the alkaline phosphate injected was about equal to that of the acid phosphate of experiment 16, suggests that the results may be explained by considering the amounts of the various substances the kidneys have to remove from the body: with only a small amount of phosphate injected the concentration ratio approaches that of urea; with a larger amount the kidney removes it with greater ease and in higher concentration than the urea; in both cases it is concentrated to a smaller extent than the creatinine. Nagayama (1920) found the urea excretion in rabbits to be more effective if phosphate was given with the urea, but this was perhaps in part due to a greater diuresis.

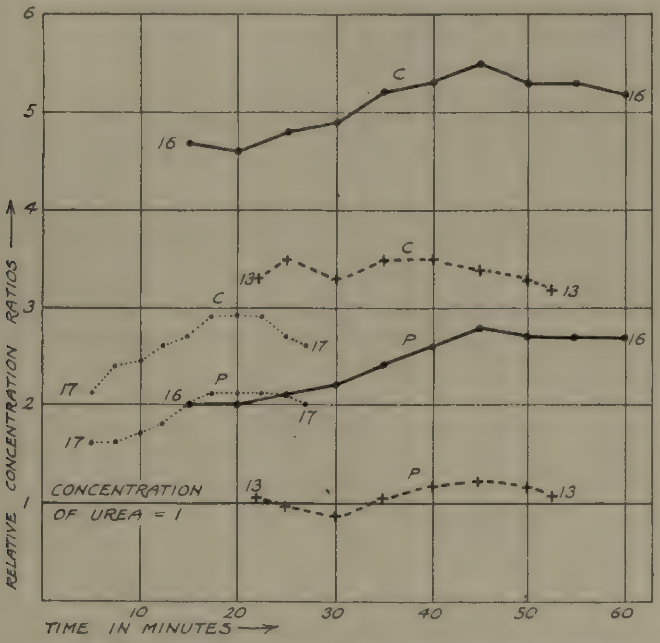

FIG. 1.—C = Creatinine. P = Inorganic phosphorus. The curves were obtained as follows: In each of the three experiments the concentrations of the different blood and urine samples were plotted along the ordinate against time of collection along the abscissa. The urine concentrations were plotted at points mid-way between the time of withdrawal of the corresponding blood-samples. Intermediate values at successive intervals of time were then read off, and the concentration ratios calculated. The values obtained are expressed relative to the concentration ratio of urea, taken as = 1. Zero time is the moment when the injection was made into the blood-stream.

It is possible that with still larger injections of phosphate the concentration ratio would approach yet closer to that of creatinine, as Mayrs (1922) found.

In Fig. 1 are plotted the concentration ratios of creatinine and inorganic phosphate relative to that of urea, which is taken as = 1, in the three experiments, 13, 16 and 17. The figure shows the varying degrees to which the three substances are concentrated; it also makes clear a general parallelism between the ratios of creatinine and inorganic phosphate, which probably depends on the rate of blood-flow through the kidney.

The fact that urea, inorganic phosphate and creatinine may be concentrated to different degrees by the kidneys during excretion is difficult of explanation on the filtration reabsorption hypothesis. It seems possible that the kidneys secrete them independently according to the requirements of the moment; and yet a fairly definite relationship between their excretion rates was clear in all experiments in which several samples of urine were obtained (*v.* Table IV). It will be seen that the amounts excreted expressed as mg. per hour, usually vary in the same direction in the different samples of a single experiment, although not exactly in equal proportions. This factor correlating the excretion rates is presumably the blood-flow through the kidney. Attempts were made to correlate the variations with variations of blood-pressure, but the relationship was by no means definite.

It was thought that some direct evidence might be thrown on the question whether urea has a "threshold," and so might be expected to be re-absorbed in the tubules, by experiments in which the formation of urea was cut down as far as possible and the amount in the blood estimated after some time. For this purpose some observations were made on animals which had been eviscerated before decerebration; the liver was left in the body, but with the structures at the portal fissure occluded by a ligature. In no case, however, was a definite result reached, the animal dying before the level of urea in the blood had fallen to any great extent. It is doubtful if this direct method of attack will be successful, since urea is possibly formed in other parts of the body than the liver (Folin and Berglund, 1922).

TABLE IV.—*Excretion Rates in Successive Samples of Urine (expressed as mg. per hour).*

Experiment No.	Urea.	Creatinine.	Inorganic phosphorus.
2	84·5	5·2	—
	46·5	3·3	—
11	72·0	6·8	—
	57·0	7·6	—
13	60·8	5·2	6·8
	166·2	15·6	18·0
	150·0	13·1	18·5
	140·0	10·8	13·2
16	55·6	17·2	27·0
	97·2	25·2	47·6
	32·6	7·4	14·4
17	267·0	48·6	162·0
	149·0	28·5	94·5
	224·4	33·0	119·4

Baglioni (1905, 1917) and Fühner (1908) have shown that urea is necessary for the proper working of the excised perfused selachian heart: it is possible that urea performs a useful function in the body of the mammal also. The experiments on the relative concentration ratios, however, lend no support to this view, as far as they bear on the question of the re-absorption of urea in the tubules of the kidney.

It was of interest to determine the rate at which the injected substances left the blood-stream: this was done by taking the blood volume as 5 per cent. of the body-weight and calculating the total amounts of the various substances in the blood before injection. Where a sample of blood was not drawn before injection, average normal values for the concentrations in the blood were taken as the basis of the calculation.

From these figures, together with the percentage amounts present in the blood after the injection, the quantity of injected substance still in the blood within a few minutes after injection could be roughly computed: the remainder must be in the tissues. Some time after the injection allowance must be made for the amounts excreted in the urine; if a large quantity of fluid was injected, an allowance could also be made for this addition to the blood volume.

The results show that within a couple of minutes after injection from 75 to 90 per cent. of the injected urea and creatinine are no longer to be found in the blood-stream: Marshall and Davis (1914) found a similar disappearance from the blood-stream on injecting large amounts of urea into dogs. Subsequently there is a further small loss to the tissues. The figures indicate that these substances leave the blood very rapidly at first, and that the rate then slows down until a balance is struck between the concentrations in the blood and in the tissues. Urea at any rate seems to be evenly distributed in the blood and the tissues throughout the body, except for the fat, where there is extremely little (Karr and Lewis, 1916; Gad Andresen, 1921). Reimann and Sauter (1921) also observed that $NaHCO_3$ injected into the dog is rapidly distributed between the blood and lymph.

The phosphate seemed to behave differently from the urea and creatinine, in that a distinctly smaller proportion left the blood-stream for the tissues, the figures varying between 65 and 80 per cent. of the total injected. This difference in their behaviour is still seen, even when allowance is made for the fact that the phosphate tends to be more concentrated in the plasma, as compared with the corpuscles, so that estimations made from plasma analyses tend to show too high a proportion of the injected substance remaining in the blood-stream.

SUMMARY.

1. In the cat the kidneys may concentrate urea, creatinine and inorganic phosphate to varying and unequal extents. The creatinine is concentrated from two to five times more than the urea; the inorganic phosphate may be concentrated to about the same extent as the urea, or up to nearly three times more. The creatinine and inorganic phosphate are not concentrated to the same degree, the former being always more concentrated by the kidney during excretion than the latter.

2. The excretion rates of urea, creatinine and inorganic phosphate show a general relationship, rising and falling together though not to the same relative degrees. The correlating factor is probably the blood-flow through the kidney.

3. After injection, urea and creatinine leave the blood-stream extremely rapidly, 90 per cent. of that injected being in the tissues within two to three minutes after completion of the injection. The rate of disappearance of inorganic phosphate is not quite so rapid, and a greater proportion remains in the blood as compared with that passing into the tissues.

4. It is considered that the results of the experiments on the relative concentration ratios of urea, creatinine and inorganic phosphate are incompatible with the "filtration reabsorption" hypothesis of kidney function in its present form. It is concluded that the kidneys must actively secrete one or more of these substances into the urine.

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REFERENCES.

Addis, T., and Watanabe, C. K.—(1917) *J. Biol. Chem.*, **29**, 391.
Baglioni, S.—(1905) *Zentralbl. f. Physiol.*, **19**, 385.—(1917) *Arch. ital. biol.*, **67**, 49.
Carr, A. D.—(1921) *J. Pharm. Exp. Ther.*, **18**, 221.
Cushny, A. R.—(1917) 'The Secretion of the Urine,' London.
Folin, O., and Berglund, H.—(1922) *J. Biol. Chem.*, **51**, 395.
Führer, H.—(1908) *Zeit. f. allg. Physiol.*, **8**, 485.
Gad Andresen, K. L.—(1921) *Biochem. Zeit.*, **116**, 268.
Haldane, J. S., and Priestley, J. G.—(1916) *J. Physiol.*, **50**, 296.
Jones, W.—(1920) 'Nucleic Acids,' London.
Karr, W. G., and Lewis, H. B.—(1916) *J. Amer. Chem. Soc.*, **38**, 1615.
Marshall, E. K., jun.—(1920) *J. Pharm. Exp. Ther.*, **16**, 141.
Marshall, E. K., jun., and Crane, M. M.—(1921) *Amer. J. Physiol.*, **55**, 278.
Marshall, E. K., jun., and Davis, D. M.—(1914) *J. Biol. Chem.*, **18**, 53.
Marshall, E. K., jun., and Kolls, A. C.—(1919) *Ibid.*, **49**, 302.
Mayrs, E. B.—(1922) *J. Physiol.*, **56**, 58.
Nagayama, T.—(1920) *Amer. J. Physiol.*, **51**, 434.
Priestley, J. G.—(1921) *J. Physiol.*, **55**, 305.
Reimann, S. P., and Sauter, M.D.—(1921) *J. Biol. Chem.*, **46**, 498.
Underhill, S. W. F.—(1923) *Brit. J. Exper. Pathol.*, **4**, 87.

A METHOD OF ESTIMATION OF DIASTASE IN BLOOD.*

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There is a general acceptance of the view that estimation of blood diastase is of distinct diagnostic value, and it is unfortunate that certain features of recent methods render them not entirely satisfactory. Stocks (1916), and later Harrison and Lawrence (1923), adopting Wohlgemuth's method, incubate a series of dilutions of blood serum, or blood plasma, with starch and obtain their results from the colour reaction which occurs on the addition of iodine. In the first place it is very difficult to obtain in general practice a sufficiency of serum for the purpose of the test (from 1 c.c. to 3 c.c.) without veni-puncture, and, in the second place, sera do occur occasionally of which the tint definitely masks the delicacy of the colour reaction. Myers and Killian (1917), using the Lewis and Benedict method of blood sugar estimation, determine the amount of sugar formed from a known quantity of starch by 2 c.c. blood after incubation for a definite period, calculating their results in terms of the percentage of starch reduced. Here again the question of veni-puncture arises, and in addition that of the inaccuracies which the picric acid methods of sugar estimation present as compared with more recent means of blood-sugar determination.

In view of these difficulties the problem of the estimation of blood diastase was approached, and, by an adaptation of MacLean's (1919) method of blood-sugar estimation, a means has been evolved which has the advantage of simplicity and accuracy, while it necessitates the minimum of discomfort to the patient. It also permits of an almost concurrent reading of the blood-sugar and the blood-diastase figures, dispensing with the need for two different techniques. For the method proposed 0·2 c.c. blood only is required—an amount which can easily be obtained by pricking the ear or the finger. The final result depends upon a determination of the patient's blood sugar by MacLean's method, and upon a second determination after 0·2 c.c. of the patient's blood has been incubated for half an hour at 37°C. with 1 c.c. of a 0·1 per cent. solution of starch.

The Effect on Blood Sugar of Incubation at 37° C.

In view of the fact that the literature of the subject gives most discordant views on the problem of glycolysis in blood, it was thought best to make an investigation of the question. It was found that under the experimental conditions described below no glycolysis was in evidence. Freshly drawn blood to the amount of 0·2 c.c. was pipetted into a small Erlenmeyer flask containing 2·8 c.c. normal saline solution. The suspension was incubated for half an hour in a water-bath at an accurately maintained temperature of 37°C. During this period a direct control estimation of the sugar in another sample of 0·2 c.c. was done. When the incubation time was completed the amount of sugar

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* Work carried out in the tenure of a Carnegie Research Assistantship.

present in the specimen was estimated. Table I shows the results in six cases taken from some forty.

TABLE I.—*The Effect of Incubation on the Sugar in 0·2 c.c. Blood.*

Case.	Control percentage.	Percentage after incubation.
1	·081	·082
2	·097	·097
3	·102	·106
4	·13	·13
5	·097	·097
6	·131	·134

No appreciable change in the sugar content occurs on incubation of 0·2 c.c. blood in 2·8 c.c. saline solution at 37° C. for half an hour. It should be stated here that throughout the experiment and also throughout the entire diastase investigation, strict aseptic precautions were observed to exclude the possibility of bacterial action during incubation.

THE PROPOSED METHOD DESCRIBED.

Into one of two 100 c.c. Erlenmeyer flasks 1·8 c.c. of 0·9 per cent. saline solution and 1 c.c. of 0·1 per cent. starch solution (*i. e.*, 1 mg. starch) is accurately pipetted, while into the other (control flask) exactly 2·8 c.c. of 0·9 per cent. saline solution is introduced. 0·2 c.c. blood is withdrawn from the finger into a special MacLean pipette* and carefully ejected *into* the fluid of the first flask, the point of the pipette being held beneath the surface of the solution, while the flask is held at an angle. The pipette is rendered free from blood by repeated washing with the clear fluid into which the blood has just been delivered. The flask is then gently shaken with a circular movement so as to mix thoroughly the blood and the solution. A second sample of 0·2 c.c. blood is similarly delivered and washed into the control flask. Both flasks, provided with rubber stoppers, fitted with capillary points, are placed in a water-bath, the temperature of which is accurately maintained at 37° C. Incubation is allowed to proceed for exactly half an hour, at the end of which time the flasks are removed and 21 c.c. of MacLean's acid sodium sulphate solution is added. In the case of the first flask the addition should be made immediately on removal from the water-bath so as to stop the action of the diastase. The subsequent steps in the estimation are precisely as described by MacLean. Briefly the treatment is as follows: The flasks are heated till the boiling-point is just reached. 1 c.c. dialysed iron is added to each and after cooling under the water tap the contents are filtered. To 20 c.c. of each filtrate is added 2 c.c. alkaline copper solution. The resulting solutions are then boiled for six minutes over a flame suitably adjusted to effect distinct boiling in one minute forty seconds. At the end of that period the flasks are immediately plunged into cold water and cooled thoroughly. 2 c.c. of 75 per cent. HCl (or H_2SO_4) are added, and after effervescence has finished and after standing for one minute with occasional agitation, the iodine content of the solutions is found by titration with N/400 sodium thiosulphate. During titration, in the case of the first flask, a variation of colour is seen ranging from dark amber to leaden and to pale blue, the final disappearance of which marks the completion of titration; in some cases when the blue colour is very faint the end-point may be rendered more distinct by the addition of a drop of 3 per cent. starch solution. The amount of sodium thiosulphate used is noted and its exact glucose equivalent ascertained from the glucose-thiosulphate table, or very much better, from a plotted graph. The second flask is treated in exactly the same manner except that starch must be added to complete the titration.

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* To be obtained from Hawksley & Son, Wigmore St., London.

Calculation.

The result is calculated as the percentage of soluble starch transformed to sugar (calculated as glucose) by the 0·2 c.c. blood employed. The amount of starch used is 1 mg., and the difference between the sugar contents, measured in fractions of a milligramme, of the two samples of 0·2 c.c. blood will be equivalent to the amount of starch reduced to sugar.

Suppose that, reading from the glucose-thiosulphate graph, the sugar content of the control preparation is 0·164 mg., while for the starch preparation it is 0·259 mg. Since the amount of filtrate used corresponds to $\frac{4}{5}$ of 0·2 c.c. blood, that amount of blood would contain 0·205 mg. sugar in the one case and 0·323 mg. sugar in the other. The difference, 0·118, is equivalent to the amount of starch transformed to sugar by incubation with 0·2 c.c. blood, so that the diastatic index in this case is 11·8. Allowance should, of course, be made for any slight reducing action of the soluble starch. During the course of this investigation the starch solutions were repeatedly tested for any such action, and in no case was it found necessary to make correction.

In view of the fact that glycolysis does not occur on incubation of 0·2 c.c. blood under the conditions of the experiment, there appears to be no good reason for running a control. The half hour during which the starch preparation is incubating may be very conveniently occupied in making a direct estimation of freshly-drawn blood.

DETAILS OF METHOD AND RESULTS.

Accuracy.—It may seem superfluous to call attention to the need for accuracy in this estimation, but in view of the fact that determinations are made in milligrammes and fractions of milligrammes, perhaps a few points which lend themselves to precision are worthy of note. Cleanliness and attention to aseptic precautions should be maintained throughout. Burettes, flasks and pipettes should be thoroughly cleaned. Antiformin or a solution of potassium bichromate in sulphuric acid is a very useful help. Standardised burettes and pipettes should be used. During the process of the boiling of the blood filtrate and the copper solution, the manometer should be carefully watched for any change in gas pressure and any necessary adjustment made.

Preparation of the starch solution.—Lintner's soluble starch was used throughout this investigation. The solvent was physiological salt solution prepared with water, double (glass) distilled and practically neutral to rosolic acid. The presence of the salt solution prevents hæmolysis, and tends to accelerate the action of diastase. A litre of 0·9 per cent. NaCl is prepared with re-distilled water, and 0·2 grm. of soluble starch weighed out and suspended in about 5 c.c. of the saline solution. Sufficient saline to make 200 c.c. is measured out and heated in a large flask almost to boiling-point, when the starch suspension is carefully added drop by drop, the contents of the flask being shaken after each addition. A reflux condenser is then attached, and the solution allowed to boil for about ten minutes. The resulting starch solution is homogeneous and transparent, and allows of an intimate association of the enzyme and substrate. It is not advisable to submit the small quantity of starch to prolonged boiling, as is recommended by certain authors (Sherman, Kendal and Clark, 1910), who, however, use a larger quantity of starch. Table II shows how the diastatic index tends to be lowered when starch which has been boiled for some time is used in the test.

TABLE II.—*Showing Variations in Diastatic Activity when* 0·2 *c.c. Blood is treated with Starch Solutions boiled for Different Lengths of Time.*

Case.	Starch solution boiled for—		
	¼ hour.	1 hour.	2 hours.
	Reducing sugar formed.		
1	·251 mg.	·230 mg.	·200 mg.
2	·180 ,,	·145 ,,	·127 ,,
3	·230 ,,	·165 ,,	·16 ,,
4	·108 ,,	·098 ,,	·08 ,,
5	·143 ,,	— ,,	·035 ,,

The starch solution should be made with great care, and should be freshly prepared every second or third day. No advantage seems to be gained in the use of glycogen instead of starch (Table III).

TABLE III.—*Showing Comparative Activity of Diastase on Starch and Glycogen.*

Case.	Blood sugar.	Sugar formed.	
		0·1 per cent. starch.	0·1 per cent. glycogen.
	per cent.	mg.	mg.
O. B—	·13	·200	·130
H. M—	·10	·087	·08
E. A—	·11	·141	·14
A. T—	·09	·093	·083
A. I—	·195	·315	·320

The diastatic figures of six normal subjects are given in Table IV. The figure seems to vary in different individuals, the average being about 8 to 10—a figure somewhat lower than that of Myers and Killian (1917), and similar to that of Stocks (1916). From data obtained it would seem that the normal index should not exceed 15.

TABLE IV.—*Showing the Diastatic Activity of Blood in Normal Cases.*

Case.	Age.	Sex.	Diastase.	Blood sugar.
R. R—	30	M.	8·5	·102 per cent.
E. A—.	22	M.	9·0	·097 ,,
A. T—	33	M.	11·8	·134 ,,
A. G—	34	M.	8·8	·102 ,,
C. R—	30	M.	7·1	·131 ,,
W. S—	30	M.	6·5	·106 ,,

Table V gives the diastatic activity of three cases of diabetes. In the case D. G—, cardiac complications and pulmonary fibrosis were present.

Table V.—*The Diastatic Activity of the Blood in three Abnormal Cases.*

Case.	Age.	Sex.	Date.	Blood diastase.	Blood sugar per cent.	Urine diastase.	Urine sugar per cent.	Diet.	Remarks.
A. I—	39	M.	13/12/22	24·5	·19	—	·5	Restricted	—
D. G—	28	M.	16/2/23	14·5	·111	6·6	·25	Not restricted	No albumin in urine.
A. C—	30	M.	8/3/23	2·375	·122	10	·66	Restricted	No albumin in urine.
				3·125	·111	12	·4		

In the course of this investigation certain points have arisen which seem to suggest that the hourly diastatic activity of the blood should not be represented by a constant figure as has been suggested by some. An investigation of the matter has already been commenced and certain observations have been made.

ADVANTAGES OF THE PROPOSED METHOD.

Sugar estimations with picric acid very frequently give results which are decidedly too high. This may be due to—

(*a*) The disturbing effect of creatinine, which varies in amount pathologically and physiologically. In certain renal conditions creatinine is found in excess. On ingestion of food it may be increased, as has been confirmed by many observers, in particular Rose and Dimmitt (1916), who showed that the excess may continue for some days after ingestion. On muscular exercise an increase may be found if measured at a short interval after exercise (Schulz, 1912). No doubt other metabolic influences may have an effect on the creatinine content.

(*b*) The presence of an unknown substance or substances in the red blood-cells, as demonstrated by De Wesselow (1919), who found marked variation in the sugar concentrations depending on the number of red blood-corpuscles, and obviously not due to creatinine.

The present method has the advantage of avoiding these sources of inaccuracy. In addition, its simplicity, rapidity and cheapness are notable considerations; the avoidance of the necessity of using such expensive apparatus as a colorimeter, as in Benedict's and Folin's methods, is important.*

I am indebted to Prof. J. A. MacWilliam and to Prof. Hugh MacLean for much kindly help and criticism.

REFERENCES.

De Wesselow, O. L. V.—(1919) *Biochem. J.*, **13**, 148.
Harrison, G. A., and Lawrence, R. D.—(1923) *Lancet*, **1**, 169.
MacLean, Hugh.—(1919) *Biochem. J.*, **13**, 135.
Myers, V. C., and Killian, J. A.—(1917) *J. Biol. Chem.*, **29**, 179.
Rose, W. C., and Dimmitt, F. W.—(1916) *J. Biol. Chem.*, **26**, 345.
Schulz, W.—(1912) *Arch. f. d. ges. Physiol.*, **186**, 726.
Sherman, H. C., Kendal, E. C., and Clark, E. D.—(1910) *J. Amer. Chem. Soc.*, **32**, 1073.
Stocks, P.—(1916) *Quart. J. Med.*, **9**, 216.

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* The necessary equipment for the present method can be obtained from Hawksley & Sons, Wigmore Street, London, for about three pounds.

THE PRECIPITATION OF COLLOIDAL GOLD BY CEREBROSPINAL FLUID; THE DIAGNOSIS OF NEURO-SYPHILIS.

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THE use of colloidal gold for the diagnosis of neuro-syphilis has not come into general use because of the difficulty of preparing a standard colloidal gold solution and the apparent unreliability of the test. In this paper we describe a new and simple method of preparing gold and specify in detail the various precautions to be adopted in the process. We also describe the conditions under which colloidal gold is precipitated by normal and pathological cerebrospinal fluids. Finally we discuss the specific mechanisms which underlie the reactions observed in the precipitation of colloidal gold by cerebrospinal fluids obtained from cases of neuro-syphilis.

The precipitation of colloidal gold by cerebrospinal fluid was first investigated by Carl Lange (1912). He found that colloidal gold was not precipitated by cerebrospinal fluids obtained from normal individuals, but that cerebrospinal fluids obtained from people suffering from certain affections of the central nervous system gave definite precipitation reactions. He observed that these precipitations invariably occurred with cerebrospinal fluids obtained from luetic affections of the central nervous system, and considered that the colloidal gold test was of great value in the diagnosis of these conditions. These observations of Lange have been extensively investigated by various observers and have been substantially confirmed.

In 1914 Miller and Levy (1914) emphasised the fact that the precipitations of colloidal gold by cerebrospinal fluids from cases of tabes and general paresis differ in a fundamental manner, and that the gold test could be used not only to differentiate luetic from non-luetic conditions, but also to distinguish tabes from general paresis. Miller, Brush, Hammers and Felton (1915) confirmed the observations of Miller and Levy. They concluded that the reaction is specific in cases of general paresis, but not absolutely characteristic of tabes or cerebrospinal syphilis. They also observed that the paretic

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curve was occasionally given by cerebrospinal fluid obtained from syphilitic patients showing no evidence of dementia. Similarly, Swalm and Mann (1915) found a typical reaction in 90 per cent. of cases of paresis, but doubted whether the reaction is entirely specific. Hammes (1917), however, regards the test as more delicate than any other as an index of pathological change in the cerebrospinal fluid. He finds a typical luetic precipitation may be obtained from cerebrospinal fluid giving a negative Wassermann, but absence of precipitation of colloidal gold by cerebrospinal fluid has never been obtained with fluids giving a positive reaction.*

The mechanism of the reaction between the colloidal gold and the cerebrospinal fluid has been the subject of many investigations. Lange assumed that the reactions were due to the production in the cerebrospinal fluid of specific proteins. Weston (1918) found that the gold precipitating substance is not the Wassermann-producing substance and can be separated from it. He assumed that the active precipitating agent in cerebrospinal fluid is a globulin since it is precipitated from solution by ammonium sulphate and is destroyed by heat. In this respect it is necessary to observe that Weston did not consider an increase in globulin alone indicative of syphilis, since such an increase is found in the cerebrospinal fluids of many patients not suffering from syphilis. Felton (1917) considered that the various types of reaction were due to the antagonistic precipitating relations of albumen and globulin, the albumen exerting an inhibitory action and the globulin a precipitating action. This hypothesis was put forward independently by Cruickshank (1920). Cruickshank made a detailed investigation into the value and mechanism of the colloidal gold test. He confirmed the conclusions of Weston that the active substance in the spinal fluid of general paretics is not dialysable and resides in the globulin fraction of the protein, but stated that it is not altered by heating to the coagulable point of protein. He observed that the various types of luetic reactions could be simulated by mixtures of globulin and albumen, the globulin acting as a precipitating agent and the albumen as a protective agent. He therefore assumed that the syphilitic reactions are in part due to the presence of albumen sufficient in quantity to partially obscure the precipitating effect of globulin, and in part to a specific alteration in the physical state of the globulin which is associated with a positive electrical charge.

PREPARATION OF COLLOIDAL GOLD.

A large number of methods of preparing colloidal gold have been described by different observers, and for each of these methods particular advantages and ease of preparation are claimed. All the methods, except that described by Black, Rosenberg and McBride (1917), fail in one essential detail—they give no definite directions for controlling the reaction of the gold solution obtained. We shall show in the following pages that a variation in the reaction of a gold solution to the extent of one thousandth normal determines whether a particular sample of cerebrospinal fluid reacts as a paretic or normal fluid.

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* Extensive references to this question are given in the papers by Miller, Brush, Hammers and Felton, and by Cruickshank (1920).

In the method which we have devised only neutral substances are used, and the gold solutions obtained, are, in every respect, remarkably uniform.

Reagents required.—(1) Freshly-distilled water obtained by re-distilling laboratory-distilled water immediately before use. The distilling flask, condenser and collecting flask should be made of hard glass.

(2) 1 per cent. gold chloride solution prepared by dissolving a 15-grain tube of gold chloride in 100 c.c. of distilled water.

(3) 1 per cent. potassium oxalate solution prepared by dissolving 1 gramme of neutral potassium oxalate in 100 c.c. of distilled water.

Method.—To 100 c.c. of distilled water 1 c.c. of the potassium oxalate solution is added and the fluid is heated to boiling-point. Whilst boiling, 1 c.c. of the gold chloride solution is added drop by drop. Within half a minute a magnificent red solution of colloidal gold is obtained.

Precautions.—(1) The water should be freshly distilled. Water left in the laboratory for 24 hours gives a gold solution of a different degree of sensitivity to that obtained from freshly distilled water (distilled water used after a 2-days' fog gave a gold solution which precipitated spontaneously). The necessity for using freshly-distilled water depends upon the fact that traces of acid from the atmosphere (carbonic or other acids) very markedly effect the aggregation of the colloidal gold particles. The gold solution contains only one part of gold in ten thousand parts of water, so that a minute percentage of acid in the water attains a high value when compared in molecular magnitude to the gold.

(2) No special precautions require to be taken with the gold chloride or potassium oxalate solutions. Stock solutions appear to keep uniform over periods of months. Further, the exact amount of potassium oxalate used is not of great significance. Quantities of potassium oxalate solution varying from 0·8 c.c. to 1·2 c.c. per 100 c.c. of distilled water do not affect the sensitivity of the resulting gold solution. There is no advantage to be gained by increasing the quantity of gold chloride beyond 1 c.c. of a 1 per cent. solution to each 100 c.c. of water. A gold sol of maximum colour intensity is obtained by using this quantity.

(3) All the glassware used should be clean and of a hard variety, especially the glass beaker in which the gold sol is prepared. It is obvious that during the process of boiling any soluble alkali in the glass will tend to pass into solution. Alkali is just as detrimental as acid to the preparation of an adequate gold sol.

Testing of cerebrospinal fluid.—Throughout the investigation the methods and nomenclature of Miller and Levy were used. 1 c.c. of 0·4 per cent. NaCl was put into each of a series of ten test-tubes. To the first tube an additional 0·8 c.c. of 0·4 per cent. NaCl was added and also 0·2 c.c. of the cerebrospinal fluid, thus making the total volume 2 c.c. After mixing, 1 c.c. of this solution was added to the second tube, thus bringing the volume of fluid in that tube to 2 c.c. After mixing, 1 c.c. of this fluid was transferred to the third tube and so on. In this way a series of (n) tubes containing 1 c.c. of approximately 0·4 per cent. NaCl and quantities of cerebrospinal fluid varying from 1/10 c.c. to $1/10 \times 2^{n-1}$ c.c. were obtained. To each of these tubes 5 c.c. of colloidal gold was added and after 24 hours the precipitation was noted. The degree of precipitation varied and was designated by the numbers 0 to 5, " 0 " implying no precipitation and " 5 " complete precipitation. Thus the precipitation numbers 5 5 5 5 4 3 2 1 0 0 mean complete precipitation in the first four tubes, *i. e.* down to a dilution of 1/80 c.c. of cerebrospinal fluid, partial precipitation of a diminishing order of magnitude in the next four tubes, and absence of precipitation in the ninth and tenth tubes.

THE PRECIPITATION OF NEUTRAL COLLOIDAL GOLD BY NORMAL AND PATHOLOGICAL CEREBROSPINAL FLUIDS.

The general results obtained by us from a large series of cerebrospinal fluids may be stated thus: (1) Normal cerebrospinal fluid never precipitates colloidal gold; (2) paretic cerebrospinal fluid always precipitates colloidal gold, the type of precipitation varying to some degree with the fluid; (3) tabetic cerebrospinal fluid sometimes precipitates colloidal gold.

We have not observed that specificity in the precipitation curves of colloidal gold by tabetic and paretic cerebrospinal fluids which are generally assumed to be characteristic of these diseases. Thus we have often tested specimens of cerebrospinal fluid obtained from definite cases of untreated tabes which gave no precipitation with colloidal gold. On the other hand, cerebrospinal fluids obtained from definite cases of general paresis sometimes gave the precipitation curves which are assumed to be characteristic of tabes. We are inclined to believe that in tabes the cerebrospinal fluid does not precipitate colloidal gold, but when the tabes is associated with paresis, especially in the early cases of tabo-paresis, then the precipitation curve assumed to be characteristic of tabes is obtained. But on this point we do not make any positive statement. A much more extensive series of investigations than we have had the opportunity to perform would be required to settle the question of a definite precipitation curve characteristic of tabes only. It may be remarked that the pathological lesions of the central nervous system are essentially different in the two diseases. In tabes we find a sclerosis of the posterior columns, in general paresis a meningo-encephalitis with an exudate round the vessels of the meninges extending into the brain itself.

The following experimental results illustrate the statement that cerebrospinal fluid obtained from definite cases of general paresis may give precipitation effects which are assumed to be characteristic of general paresis or tabes:

Case.	Precipitation numbers.
1.	5 5 5 5 5 4 4 3 2 1
2.	5 5 5 5 5 4 3 2 1 0
3.	5 5 5 5 4 3 2 1 0 0
4.	4 5 5 3 3 2 1 1 0 0
5.	3 4 5 5 4 4 3 2 1 0
6.	4 5 5 5 5 5 4 3 3 2
7.	3 3 3 5 5 5 5 4 3 2

All these patients showed well-marked signs of general paralysis. It may be observed, however, that only cases (1), (2) and (3) give the precipitation numbers assumed to be typical of general paresis. The precipitation numbers of cases (4) and (5) approximate to those assumed by Miller and Levy to be characteristic of tabes, whilst the precipitation numbers of cases (6) and (7) suggest cerebrospinal syphilis. In our discussion on the mechanism of the reaction between colloidal gold and cerebrospinal fluid we come to the conclusion that the particular effects observed may be most readily explained by assuming that in pathological cerebrospinal fluid there is an increase in euglobulin, a normal constituent of cerebrospinal fluid, the degree of precipitation of the gold being proportionate to that increase. Since the lesions of tabes are fundamentally different from those of general paresis, it is difficult to appreciate the hypothesis that tabes is characterised by a small increase in the euglobulin of the cerebrospinal fluid, whilst general paresis is accompanied by a large increase in that substance. On the other hand, the presence of a small excess of euglobulin in early cases of general paresis and a large excess in late cases would readily explain the particular types of precipitation curves which have been assumed to be characteristic of tabes and general paresis. On this assumption the cerebrospinal fluid obtained from uncomplicated cases of tabes would not precipitate neutral colloidal gold, whilst cases of tabo-paresis, *i. e.* tabes going on to general paresis, would give the precipitation numbers characteristic of a small increase in the cerebrospinal fluid of euglobulin.

THE PROPERTIES OF THE PRECIPITATING SUBSTANCES.

(a) *Dialysis.*—Contradictory results have been obtained by different observers regarding the capacity of the substances concerned in the precipitation of colloidal gold to pass through parchment or collodion membranes. Cruickshank (1920) definitely found that dialysis in thin collodion membranes did not diminish the capacity of the cerebrospinal fluid to precipitate colloidal gold. On the other hand, Miller, Weston, and Matzkiewitsch (1914) all stated that the precipitating substance is dialysable. In view of the importance of the property in the analysis of the mechanism of the reaction we repeated the experiments.

Paretic cerebrospinal fluid was placed in a small collodion tube and dialysed against ·9 per cent. NaCl. The thickness of the collodion tube was such that equilibrium of the electrolytes occurred within fifteen minutes. After twenty-four hours' dialysis the following results were obtained:

Original cerebrospinal fluid .	5 5 5 5 5 5 4 3 3 0
Dialysed cerebrospinal fluid . .	5 5 5 5 5 5 4 3 3 0

The results fully confirm those of Cruickshank.

It is obvious that the precipitating substance shows no tendency whatever to pass through a collodion membrane even after prolonged dialysis, thus disproving the assumption of Matzkiewitsch (1914) that the active substance is a peptone.

(b) *Destruction by high temperature.*—A detailed investigation into the effect of heat on the capacity of a cerebrospinal fluid to precipitate colloidal gold gives interesting results. In fresh cerebrospinal fluid partial destruction of the active agent is produced by heating to 50° C. for five minutes, but this destruction is not complete even after heating to 80° C. for the same length of time. Heating to 100° C. produced complete destruction.

The following figures illustrate this statement:

Original fluid	5 5 5 5 4 3 2 1 0 0
After heating to 50° C. for 5 mins.	4 4 4 3 3 2 1 0 0 0
,, ,, 60° C. ,, ,,	4 4 4 3 2 2 1 0 0 0
,, ,, 70° C. ,, ,,	3 3 4 3 2 1 1 0 0 0
,, ,, 80° C. ,, ,,	1 1 2 1 1 0 0 0 0 0
,, ,, 100° C. ,, ,,	0 0 0 0 0 0 0 0 0 0

There is no indication from these figures of a definite temperature of destruction by heat such as is usually assigned to the heat of coagulation of a protein.

Another fact which often appears in this kind of experiment is the tendency of the precipitation curve of a heated fluid to take on a tabetic type. This is illustrated by the following results:

Original fluid	3 3 4 4 4 4 3 3 2 1
Heated to 80° C. for 5 mins.	1 2 3 5 5 4 4 3 2 0
,, 90° C. ,, ,,	0 0 2 3 3 2 1 0 0 0
,, 100° C. ,, ,,	0 0 0 0 0 0 0 0 0 0

The divergent results obtained by different observers on the effect of heat on cerebrospinal fluid are probably due to the time which has elapsed between the withdrawal of the fluid from the body and the heating experiments. Although the precipitating substance is very stable and persists in cerebrospinal fluids for a considerable period of time (months), yet old cerebrospinal fluid has its precipitating property destroyed by heating to 60° C. for 5 minutes. This result is probably due to the increased alkalinity of old cerebrospinal fluid owing to the evolution of CO_2 and the consequent formation of Na_2CO_3 from $NaHCO_3$ in the fluid. The following figures show the influence of reaction on the destruction of the precipitating substance by heat. The original unheated fluid gave the precipitation numbers 5 5 5 5 5 4 3 2 1 0. Heated to 80° C. for five minutes:

Original fluid	Reaction pH 7·6	0 0 0 0 0 0 0 0 0 0
,, ,, plus acid	,, pH 7·1	1 1 1 1 1 0 0 0 0 0
,, ,, ,, ,,	,, pH 6·5	2 5 5 5 2 0 0 0 0 0

In a definitely alkaline medium complete destruction occurred at 80° C.; in a neutral medium a small quantity of the active substance remained undestroyed; and in an acidic medium a quantity of active substance capable of giving a definite tabetic precipitation curve was left. It may be observed that the quantities of acid used had no effect on the precipitation of the colloidal gold.

(c) *Precipitation by ammonium sulphate.*—We find, in agreement with other observers (Weston, Cruickshank), that the active agent contained in paretic cerebrospinal fluid concerned in the precipitation of colloidal gold is completely precipitated by half saturation with ammonium sulphate. Thus the following reactions were given by (*a*) paretic cerebrospinal fluid, and (*b*) the solution obtained by precipitating 2 c.c. of the same cerebrospinal fluid with half saturated ammonium sulphate, and redissolving the precipitate in 2 c.c. of water:

Original fluid	5 5 5 5 4 3 2 1 0 0
Solution of Am_2SO_4 ppt.	5 5 5 5 5 5 4 3 3 2

The augmented effect which is invariably given by the ammonium sulphate precipitate suggests that a substance inhibiting the precipitation of colloidal gold is contained in cerebrospinal fluid, and that this inhibitory substance is not completely precipitated by half saturation with ammonium sulphate.

(d) *Precipitation by alcohol.*—It has been shown by Mellanby (1907) that alcohol at 15° C. not only precipitates protein from serum but renders it insoluble. If, however, the alcohol and the serum be first cooled to 0° C. before mixing, then the protein so precipitated is readily soluble in water. On adding alcohol, cooled to − 10° C., to the extent of 50 per cent. to a paretic cerebrospinal fluid, we found that the active agent was completely precipitated, and when redissolved in water gave a solution possessing an activity slightly greater than that of the original fluid:

Paretic cerebrospinal fluid	5 5 5 5 5 4 3 2 1 0
50 per cent. alcohol ppt. in water	5 5 5 5 5 5 5 4 4 0

Apparently, after alcohol precipitation also, some inhibitory substance remains in the filtrate in a manner comparable to that observed with ammonium sulphate precipitation.

The hypothesis that a substance inhibiting the precipitation of colloidal gold is present in cerebrospinal fluid, and that this substance is not readily precipitated by ammonium sulphate or alcohol, was investigated more closely in the case of precipitation by alcohol.

4 c.c. of alcohol, cooled to − 5° C., were added to 2 c.c. of cerebrospinal fluid. The precipitate was obtained by spinning the mixture in a centrifuge. This precipitate was dissolved in 2 c.c. of 0·9 per cent. NaCl. The following give the precipitation numbers of (A) the original cerebrospinal fluid, (B) the precipitate dissolved in 0·9 per cent. NaCl, (C) the filtrate freed from alcohol and made up to the original volume of cerebrospinal fluid, and (D) the fluid produced by mixing quantities of (B) and (C) equal to those contained in the original cerebrospinal fluid:

(A) Cerebrospinal fluid	3 4 5 4 3 2 1 0 0 0
(B) Alcohol precipitate	5 5 5 4 3 2 1 0 0 0
(C) Filtrate freed from alcohol	0 0 0 0 0 0 0 0 0 0
(D) Mixtures of (B) and (C) in equivalent proportions	3 4 4 4 3 2 1 0 0 0

The results show definitely (1) that the active agent is entirely precipitated unimpaired by cold alcohol, and (2) that some inhibitory substance contained in cerebrospinal fluid is not precipitated by alcohol. The results (A) and (B) show how readily a so-called tabetic precipitation curve becomes a paretic curve.

FACTORS WHICH DETERMINE THE SENSITIVITY OF COLLOIDAL GOLD TO PRECIPITATION BY CEREBROSPINAL FLUIDS.

We have never observed the precipitation of *neutral* colloidal gold by cerebrospinal fluid obtained from a normal individual. The sensitivity of a gold sol, however, is profoundly influenced by its reaction. Therefore a normal cerebrospinal fluid may give a tabetic or paretic precipitation curve with a sensitised gold solution. Similarly, a paretic fluid may give no precipitation with a desensitised gold sol and so similate a normal fluid. Generally stated, neutral salts have very little influence on colloidal gold, but the tendency to precipitate with cerebrospinal fluid is greatly augmented by traces of acid and depressed by traces of alkali.

(1) *Potassium oxalate.*—Varying amounts of potassium oxalate have very little influence on the precipitation of colloidal gold by cerebrospinal fluid, provided that the amount of oxalate used in the preparation of the colloidal gold is less than that required to produce a turbid preparation. Three gold sols were prepared in the way previously described, except that in the first case 0·8 c.c. of potassium oxalate (1 per cent.) had been added to each 100 c.c. of distilled water, in the second case 1·0 c.c. of oxalate, and in the third case 1·6 c.c. of oxalate. These three gold sols were perfectly clear and of a characteristic red colour. The precipitation numbers with the same paretic cerebrospinal fluid were:

Neutral gold containing	0·8 c.c.	pot. oxalate (1%)	per	100 c.c	5 5 5 5 4 3 2 1 0 0
,,	1·0 c.c.	,,	,,	,,	5 5 4 4 3 2 2 1 0 0
,,	1·6 c.c.	,,	,,	,,	5 5 4 4 3 3 2 1 0 0

Double the amount of potassium oxalate contained in the gold sol diminished to a very small degree only its precipitation by paretic cerebrospinal fluid.

(2) *Acid.*—Acidic colloidal gold in contra-distinction to neutral gold is readily precipitated by *normal* cerebrospinal fluid, the degree and type of precipitation varying with the reaction of the gold. The experimental results given illustrate this statement.

Neutral colloidal gold was acidified to varying degrees by the addition to it of normal oxalic acid. *Normal* cerebrospinal fluid gave the following precipitation numbers of these acidified gold sols:

Neutral gold sol	0 0 0 0 0 0 0 0 0 0	(A)
Sol containing N/5000 acid	0 0 4 3 2 1 1 0 0 0	(B)
,, ,, N/3000 ,, ...	2 4 5 5 4 2 1 0 0 0	(C)
,, ,, N/2500 ,, ...	5 5 5 5 3 2 1 0 0 0	(D)

According to the classification of Miller and Levy the absence of precipitation (A) is characteristic of normal cerebrospinal fluid, (B) simulates early tabes; (C) advanced tabes and (D) general paresis. In fact, if the test had been made with a cerebrospinal fluid of unknown origin, results indicating a normal, tabetic or paretic individual would be obtained if the reaction of the gold sol varied so little as N/2500 acid. The value of the colloidal gold test, therefore is absolutely dependent upon the neutrality of the gold sol used. This fact emphasises the necessity of using a method for the preparation of a gold sol such as we have described, *i.e.* by the use of reagents neutral in reaction. A second fact is evidenced from the above results—normal cerebrospinal fluid contains all those substances which are responsible for the peculiar precipitation effects which are assumed to be pathognomonic of neuro-syphilis.

(3) *Alkali.*—Just as acid increases the sensitivity of colloidal gold towards precipitation by cerebrospinal fluid, so alkali diminishes this tendency. The degree of diminution with alkali is of the same order as the increase observed with acid, except that only minute quantities of alkali may be added without producing spontaneous precipitation of the gold sol. The following figures give the precipitation numbers of colloidal gold to which varying small quantities of alkali had been added by a paretic cerebrospinal fluid;

Reaction of gold.	
Neutral	5 5 5 5 5 4 3 2 2 0
N/50,000 alkali	4 5 5 5 4 4 3 2 2 0
N/25,000 ,,	4 4 4 4 3 2 2 1 0 0
N/10,000 ,,	4 3 2 2 1 1 1 1 0 0

It may be observed that the quantities of alkali are only one-tenth of the quantities of acid used in a corresponding experiment. From these figures we see that minute quantities of alkali convert a paretic into a tabetic precipitation curve, and larger quantities a paretic into practically a normal curve. In fact, just as a gold sol sensitised with acid can give a paretic precipitation curve with a normal cerebrospinal fluid, so a gold sol desensitised with alkali can show no precipitation with a paretic cerebrospinal fluid. The results again emphasise the necessity of working with a neutral gold sol.

(4) *Age.*—Although it is absolutely essential to use freshly distilled water to prepare colloidal gold, yet, when the gold sol has been prepared, the time which has elapsed between its preparation and the testing of a fluid on it is of minor importance. It is necessary to keep the gold sol in a stoppered bottle of hard glass, otherwise spontaneous precipitation occurs. A gold sol left in contact with the air becomes more sensitive to precipitation, probably owing to the solution in it of acids (CO_2, etc.) from the atmosphere. This statement is illustrated by the following results:

Fresh gold sol with normal C.S.F.	0 0 0 0 0 0 0 0 0 0
Same gold sol 24 hours later with same C.S.F.	0 1 3 4 3 2 1 0 0 0

During the 24 hours in which the gold sol was left open to the air the atmosphere was humid and foggy. Possibly as a routine practice it is better to use freshly prepared colloidal gold.

THE PRECIPITATION OF COLLOIDAL IRON BY CEREBROSPINAL FLUID.

An analysis of the precipitation numbers of colloidal gold by luetic cerebrospinal fluids indicates that in these reactions not only a precipitating agent but also an anti-precipitating agent is involved. On the accepted hypothesis of colloidal solutions the gold sol is precipitated by a positively charged colloid contained in the cerebrospinal fluid. It appeared possible that the substances inhibiting precipitation might be the inorganic salts contained in the cerebrospinal fluids. Quantitative experiments, however, showed that electrolytes, even in large amounts, show little tendency to inhibit the precipitation of antagonistic colloids. They tend rather to bring one colloid out of solution and so render the second colloid more unstable. It appeared probable, therefore, that the inhibitory substance was a second colloid. This hypothesis was confirmed by the observation that cerebrospinal fluids precipitate positively charged colloidal iron in definite dilutions. The following experimental result illustrates this statement:

Colloidal iron (0·05 per cent.) was substituted for colloidal gold in a test made in the usual way on paretic cerebrospinal fluid. The precipitation numbers for the colloidal iron were 5 5 5 0 0 0 0 0 0 0. The precipitation numbers for the same cerebrospinal fluid in colloidal gold were 5 5 5 5 5 5 4 3 3 0. It may be assumed, therefore, that this cerebrospinal fluid contained not only positively charged colloid in relatively large quantities, which precipitated colloidal gold, but also a negatively charged colloid in smaller concentrations which precipitated colloidal iron. It may be asked why these two oppositely charged colloids do not produce mutual precipitation in cerebrospinal fluid. The absence of precipitation is probably due to the colloid which is present in excess keeping the other colloid in solution just as comparatively large quantities of colloidal iron keep colloidal gold in solution.

THE MUTUAL PRECIPITATION OF ANTAGONISTIC COLLOIDS.

The factors involved in the precipitation of colloidal gold by luetic cerebrospinal fluids may be analysed by determining (1) the precipitation of colloidal gold by colloidal iron, and (2) the precipitation of colloidal gold by albumen, globulin and serum.

(1) *The Mutual Precipitation of Colloidal Gold and Colloidal Iron.*

Colloidal gold carries a negative charge, colloidal iron carries a positive charge. Therefore in suitable dilutions these two colloidal solutions mutually precipitate one another. The quantitative relations involved in these precipitations were determined by precipitating colloidal gold by colloidal iron in a manner similar to that used in the cerebrospinal fluid tests, *i. e.* colloidal iron 0·5 per cent. was substituted for the cerebrospinal fluid. The quantity of colloidal gold used was constant (5 c.c.); the quantities of colloidal iron and the degrees of precipitation are shown:

Colloidal gold (0·01 per cent.) in each tube, 5 c.c.:

Colloidal iron (0·5 per cent.) .	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$
Degree of ppt. . . .	0	0	0	0	5	5	5	5	5	0

From these figures it may be seen that approximately 0·00005 grm. of colloidal iron completely precipitate, 0·0005 grm. of colloidal gold, and that this precipitation continues until the iron is reduced to 0·000002 grm. Quantities of colloidal iron greater than 0·0005 grm. keep the gold in solution and 0·0005 grm. of colloidal gold keep quantities of iron less than ·000002 grm. in solution. In fact, on adding continually diminishing quantities of colloidal iron to a constant quantity of colloidal gold three zones may be observed: (a) the first zone in which the iron keeps the gold in solution, (b) the second zone in which all the iron and all the gold is precipitated, the colour of the precipitate varying from brown when the iron is relatively large in quantity to purple when the gold predominates, and (c) a zone in which the gold keeps minute quantities of iron in solution. There is no definite chemical relation between the amount of iron and gold precipitated. It is obvious, however, that the effects observed in the precipitation of neutral colloidal gold by neutral colloidal iron are not comparable to the precipitation phenomena obtained when colloidal gold is precipitated by a luetic cerebrospinal fluid. In fact we cannot assume that the sole agent involved in the precipitation of colloidal gold by luetic cerebrospinal fluids is a positively charged colloid.

(2) *The Precipitation of Colloidal Gold by Emulsoid Colloids.*

Colloidal gold is not precipitated by egg-albumen, gelatine, starch or glycogen in any dilution. Presumably therefore these emulsoid colloids carry a negative charge similar to that carried by colloidal gold. Precipitation, however, is produced by very small quantities of "eu" globulin when dissolved in 0·9 per cent. NaCl, and to a less degree by this protein when present in serum.

(a) *Euglobulin.*—The precipitation of colloidal gold by euglobulin is broadly similar to the precipitation of gold by iron. Small quantities of globulin give the typical paretic precipitation curve; larger quantities tend to keep the gold in solution.

Serum globulin was precipitated by diluting serum with ten volumes of distilled water and bringing the mixture to the iso-electric point for globulin by the cautious addition of acetic acid. The precipitated globulin was obtained as a compact mass by spinning the fluid, and, after pouring off the supernatant liquid, was dissolved in a volume of ·9 per cent. NaCl equal to that of the original serum. It may be observed that the globulin so obtained is "eu" globulin only, and not a mixture of "eu" and "pseudo" globulin, which is obtained on half saturating serum with ammonium sulphate. This globulin solution diluted to one part in a thousand parts of salt solution gave the following precipitation numbers:

0·1 per cent. of globulin solution . . . 5 5 5 4 3 2 1 0 0 0

The actual strength of globulin solution used was 0·0005 per cent., so that, from the above figures, 0·0000001 grm. of globulin precipitates completely 5 c.c. of colloidal gold. It is interesting to observe that a minimal quantity of 0·000002 grm. of colloidal iron also completely precipitates 5 c.c. of colloidal gold—an amount which is twenty times as great as the globulin. More concentrated solutions of globulin gave precipitation numbers which indicate that euglobulin in relatively large quantities tends to hold gold in solution in a manner comparable to that observed with colloidal iron. Thus 1 per cent. of globulin solution gave the precipitation numbers 3 3 3 5 5 5 4 0 0 0. The capacity of globulin to keep gold in solution is not equal to that shown by colloidal iron. We discuss this question more fully when we consider the general questions involved in the precipitation curves of luetic cerebrospinal fluids.

(b) *Serum.*—The precipitation of colloidal gold by serum appears to be due entirely to the euglobulin contained within it. But it is evident that some substance inhibiting the precipitation of colloidal gold by euglobulin is also present in serum, as shown by the following precipitation figures for serum:

(A) Serum 0·1 per cent.	4 4 3 3 0 0 0 0 0 0
(B) Euglobulin from a comparable volume of serum	5 5 5 4 3 2 0 0 0 0
(C) "Eu" and "pseudo" globulin from a comparable volume of serum . . .	4 4 3 3 0 0 0 0 0 0

From (A) and (B) it may be seen that euglobulin prepared from serum is a more effective precipitant than the serum itself—that is, that serum contains some substance which inhibits the precipitation of colloidal gold by euglobulin. The result (C) shows that the precipitate obtained from serum by half saturation with ammonium sulphate, when redissolved in water, has the same precipitation effect as the original serum. Therefore this precipitate contains not only the precipitating agent but also the anti-precipitant. Broadly speaking, the two proteins precipitated from serum by half saturation with ammonium sulphate are "eu" and "pseudo" globulin. Euglobulin in minute quantities precipitates colloidal gold. Therefore we must assume that pseudoglobulin, or some substance associated with the pseudoglobulin in its precipitations, is the agent contained in serum which inhibits precipitation.

DISCUSSION OF RESULTS.

(1) The facts observed in the precipitation of colloidal gold by cerebrospinal fluid may be briefly summarised:

(*a*) Neutral colloidal gold is not precipitated by normal cerebrospinal fluid, but gives typical precipitation curves with luetic cerebrospinal fluid.

(*b*) Colloidal gold which has been sensitised by the addition of acid gives a tabetic or paretic precipitation curve with normal cerebrospinal fluid according as the quantity of acid added, and therefore the degree of sensitisation is small or large. Similarly, colloidal gold which has been desensitised by the addition of alkali gives a tabetic precipitation curve with paretic cerebrospinal fluid. From this it follows that the precipitating agents concerned in the luetic reactions are contained in a subminimal quantity in normal cerebrospinal fluid and in increasing quantities in tabetic and paretic cerebrospinal fluids.

(*c*) The precipitating agents are nondialysable, precipitated unchanged by half saturation with ammonium sulphate or by cold alcohol, and are slowly destroyed by heat at temperatures depending upon the reaction of the cerebrospinal fluid. These facts indicate that the precipitating factors are either "eu" or "pseudo" globulin, or a mixture of them.

(2) The precipitation of colloidal gold by oppositely charged colloids such as colloidal iron or euglobulin may be summarised:

(*a*) Colloidal iron or euglobulin totally precipitates gold over a large range of concentrations, but the precipitation is mutual and complete over this range.

(*b*) Large quantities of iron or globulin keep the gold in solution, and, similarly, colloidal gold keeps minute quantities of colloidal iron or globulin in solution. In fact, a colloid is not precipitated by an oppositely charged colloid if present in relatively large quantities.

A comparison of the results summarised in (1) and (2) appears to indicate that the phenomena observed in the precipitation of colloidal gold by luetic cerebrospinal fluid are completely analogous to the mutual precipitations of antagonistic colloids. But the similarity is only superficial, for whereas large quantities of colloidal iron keep colloidal gold in solution, the typical tabetic curve is obtained intermediate between the absence of precipitation by normal cerebrospinal fluid and full precipitation by paretic cerebrospinal fluid. In other words, normal cerebrospinal fluid contains only a trace of the precipitating agent and produces no observable effect on neutral colloidal gold, tabetic fluid contains a larger quantity of this substance and gives its characteristic curve with precipitation in the middle tubes, whilst paretic fluid contains the greatest quantity and gives complete precipitations at high concentrations.

The presence of antagonistic colloids in cerebrospinal fluid is evident, since this fluid precipitation not only negatively charged colloidal gold, but also positively charged colloidal iron. It is probable, therefore, that the phenomena observed in the precipitation of colloidal gold by luetic fluids may be explained on the assumption of a balanced reaction between these oppositely charged colloids. This assumption is supported by the observations made on the precipitation of colloidal gold by (*a*) euglobulin and (*b*) a mixture of "eu" and "pseudo" globulin. The results so obtained may be appreciated if it is assumed that euglobulin actively precipitates colloidal gold, whilst pseudoglobulin tends to keep gold in solution. From a consideration of these facts we put forward the following hypothesis: All cerebrospinal fluids contain "eu" and "pseudo" globulin, the quantities of these substances increasing as we pass from normal to tabetic and from tabetic to paretic cerebrospinal fluid. "Eu" globulin is the active agent which precipitates colloidal gold; "pseudo" globulin tends to keep the gold in solution and antagonises the precipitating action of "eu" globulin. "Eu" globulin, being soluble only in electrolytes, tends to come out of solution on dilution, and therefore its precipitating action is augmented on dilution; "pseudo" globulin, on the other hand, is soluble in water, and therefore its capacity to hold gold in solution is diminished by dilution in a magnitude equal to the degree of dilution. The increased precipitating action of euglobulin on dilution appears to be responsible for the precipitation curve which has been described as typical of tabes. Probably all cerebrospinal fluids contain a constant quantity of "pseudo" globulin, but variable quantities of "eu" globulin. The tabetic curve is due to a small increase in "eu" globulin, which manifests its action only in the intermediate dilutions when its tendency to come out of solution is augmented to a maximal extent by dilution. In general paresis, on the other hand, the "eu" globulin is increased to such an extent that it overcomes the inhibitory action of the "pseudo" globulin and precipitates colloidal gold in the first dilutions of the gold sol test.

It is possible, however, that "pseudo" globulin as well as "eu" globulin may be increased in certain luetic conditions. Thus in cerebral syphilis the cerebrospinal fluid has been stated to give the following precipitation numbers —0 0 0 5 5 5 4 3 2 1. On our hypothesis the absence of precipitation in the first tubes would be due to an increased quantity of "pseudo" globulin preventing the precipitation of the gold by the "eu" globulin, but on increasing dilution the "eu" globulin tends to come out of solution, and this increased tendency to precipitation overcomes the inhibitory action of the "pseudo" globulin and precipitations in high dilutions result. This explanation is more probable than the assumption that cerebral syphilis is associated with a large increase of "eu" globulin in the cerebrospinal fluid. "Eu" globulin has to be present in massive quantities before it can keep gold in solution.

Finally, we propose to discuss at a later date the question whether the "eu" globulin found in syphilitic cerebrospinal fluids, although similar in physical properties to the "eu" globulin of serum, yet differs from it in chemical composition, and is a specific result of the action of the syphilitic virus.

SUMMARY.

(1) Colloidal gold, neutral in reaction, may be prepared by adding 1 c.c. of $AuCl_3$ (1 per cent.) to 100 c.c. of boiling potassium oxalate (0·01 per cent.).

(2) Precipitation of neutral colloidal gold is always produced by paretic cerebrospinal fluid, sometimes by tabetic fluid, but never by normal fluid.

Cerebrospinal fluid obtained from definite cases of general paresis often gives the precipitation curve assumed to be characteristic of tabes.

(3) The precipitating substance is (*a*) non-dialysable, (*b*) precipitated by half saturation with ammonium sulphate, (*c*) precipitated by 50 per cent. alcohol at 0° C., (*d*) destroyed by heat at temperatures depending upon the reaction of the fluid and the duration of heating.

(4) Neutral colloidal gold, sensitised by the addition of acid, may give a tabetic or paretic precipitation curve with normal cerebrospinal fluid depending on the degree of sensitisation, and *vice versâ* on desensitisation by alkali. These facts emphasise the necessity of working with a neutral colloidal gold.

(5) Neutral salts have a small influence only on the sensitivity of colloidal gold to precipitation by cerebrospinal fluid.

(6) All cerebrospinal fluids contain not only a positively charged colloid which precipitates colloidal gold, but also a negatively charged colloid which precipitates colloidal iron, and therefore antagonises the precipitating action of the former on a gold sol. The positive colloid appears to be a "eu" globulin, the negative colloid a "pseudo" globulin. In normal cerebrospinal fluids the "eu" and "pseudo" globulins are present in small quantities only, and the precipitating action of the "eu" globulin is balanced by the inhibitory action of the "pseudo" globulin unless a gold sol is used which has been sensitised by acid (*i.e.* a sol in which the tendency of the gold to precipitate has been augmented). In cerebrospinal fluids which give the tabes precipitation curve the "eu" globulin is increased in amount to a moderate extent, and to a larger extent in fluids which give the paretic precipitation curve. Possibly the precipitation curves stated to be characteristic of cerebrospinal syphilis, *i.e.* absence of precipitation in low dilutions and complete precipitation in high dilutions, are due to a large increase of both "pseudo" and "eu" globulins in the cerebrospinal fluid.

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REFERENCES.

Black, J. H., Rosenberg, L., and McBride, R. B.—(1917) *J. Amer. Med. Assoc.*, **69**, 1855.

Cruickshank, J.—(1920) *Brit. J. Exper. Pathol.*, **1**, 71.

Felton, L. D.—(1917) *New York Med. J.*, **105**, 1170.

Hammes, E. M.—(1917) *Amer. J. Med. Sci.*, **154**, 625.

Lange, C.—(1912) *Berlin. klin. Wchnschr.*, **49**, 897.

Matzkiewitsch, J.—(1914) *Deutsche med. Wchnschr.*, **40**, 1221.

Mellanby, J.—(1907) *J. Physiol.*, **36**, 288.

Miller, S. R., and Levy, R. L.—(1914) *Johns Hopkins Hosp. Bull.*, **25**, 133.

Miller, S. R., Brush, N. D., Hammers, J. S., and Felton, Ll. D.,—(1915) *Ibid.*, **26**, 391.

Swalm, C. J., and Mann, A. L.—(1915) *New York Med. J.*, **101**, 719.

Weston, P. G.—(1918) *Amer. J. Insan.*, **74**, 431.

SOME NEW PRINCIPLES IN BACTERIAL IMMUNITY THEIR EXPERIMENTAL FOUNDATION, AND THEIR APPLICATION TO THE TREATMENT OF REFRACTORY INFECTIONS.

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GENERAL STATEMENT OF THEORIES AND RESULTS.

In spite of a great accumulation of experimental and clinical data concerning the principles and the practical application of bacteriotherapy, serotherapy, and prophylaxis, the therapeutic results obtained, magnificent though they may often be, are strangely lacking in uniformity.

The effects, for example, of specific vaccines upon acute or chronic infections may be in one case almost uniformly favourable, and, in another, completely negative. The same statements apply also to the prophylactic and therapeutic bacterial anti-sera.

Now, when one reviews this field of experience, and searches for possible reasons for the lack of uniform results, one is struck by the fact that, in general, the successes of bacterial therapy and prophylaxis are obtained with micro-organisms of the non-acid-fast and Gram-negative varieties. Failure, on the contrary, is the general rule where acid-fast and Gram-positive bacteria are in question. It must be understood that we are here considering only the bacterial bodies themselves, and not their soluble products, such as toxins. It is true that we cannot claim for this general principle the validity of a clear-cut and universal law, for there are certain cases in which the rule does not altogether hold good: for example, partial successes have been obtained with pneumococcal antiserum, and with staphylococcal vaccines; and, on the contrary, failure is very far from rare with vaccines of Gram-negative bacteria such as the gonococcus.

Nevertheless the rule holds good in a sufficiently large proportion of cases to give us an important clue towards the discovery of the underlying truth.

Since acid-fastness and Gram-positiveness have been clearly proved to be associated with the presence in the bacterium of certain waxy, fatty or lipoid substances,* it seemed probable, *à priori*, that these substances were the cause of failure in immunisation. Owing to their power of protecting the specific bacterial proteins and preventing their liberation from the body of the bacterium, these substances could be conceived as preventing or retarding the production of the sole adequate stimulus for the immunity reaction of the infected organism.

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* In the remainder of this paper the waxy, fatty or lipoid substances of bacteria of all varieties will be referred to as "lipoidal" substances.

Support for this view is to be found in the work of Douglas (1921) on the tryptic digestion of acetone-extracted bacteria. He had invariable success in digesting and obtaining immunity responses with Gram-negative bacteria, such as the dysentery bacillus, the typhoid bacillus and the gonococcus, whereas the Gram-positive organisms either failed altogether to digest (staphylococcus) or underwent apparent digestion, but gave rise to no immune bodies (*B. welchii*).

In view of these circumstances it seemed desirable to attack the problem by attempting the removal, by extraction, of the "lipoidal" fraction of some representative acid-fast and Gram-negative bacteria. The tubercle bacillus was first selected, as having a highly-developed lipoid constituent, and as being, in many respects, a very robust and resistant organism.

A number of processes involving simple extraction with various fat-solvents were unsuccessfully tried. For instance, a large quantity of *B. tuberculosis* was killed with heat, dried, ground up and extracted with acetone in a Soxhlet apparatus for 34 days without intermission at 65° C. At the end of this time, although large quantities of "lipoidal" substances had been extracted, the bacilli were found to be still quite acid-fast.

It thus became clear that something more than simple extraction with ordinary fat-solvents was necessary if the removal of the acid-fast substances was to be effected.

Fortunately a clue to the problem was forthcoming in the shape of the following fact, which had been observed by various workers, including myself, viz. that a tuberculous tissue fixed with formalin often tends to show far fewer tubercle bacilli, when suitably stained, than the same tissue fixed with perchloride of mercury.

Might it not be, then, that formalin removes the acid-fast substances, and thus prevents the demonstration of the bacilli by staining?

To test this hypothesis, tubercle bacilli, grown on the usual media, were treated with strong formalin for various lengths of time, but the result appeared negative, for the bacilli remained acid-fast, although their appearance underwent a definite change in the direction of increased granularity.

Evidently, then, it could not be the formalin alone that caused the loss of acid-fastness. In the preparation of tissues for section, however, a number of other reagents are employed, including fat-solvents such as alcohol, xylol or chloroform. It therefore seemed to me probable that the phenomenon might be due to the successive application of formalin and fat-solvents to the bacilli.

Experiments at once showed that, with a suitable technique, the tubercle bacillus could readily be rendered non-acid-fast in this manner.

Now, in view of our knowledge that bacteria killed with formalin retain their antigenic power, as they do also after simple extraction with acetone (Douglas), there was good reason to hope that the residue of the tubercle bacillus, after treatment with formalin and extraction with acetone, would retain all or part of the specific properties of the bacillus, and that this residue could be used for immunisation. Numerous experiments demonstrated the correctness of this reasoning, as will be seen later.

The facts so far established led me to the expectation that it would be possible to deal with other acid-fast bacteria in the same manner, and also, by a similar process, to reduce Gram-positive micro-organisms to the Gram-negative condition. This was found to be the case, and it was further shown that in this state they no longer resisted the digestive action of trypsin.

The next step achieved was the demonstration of the capacity of the "defatted" bacteria to react with specific antisera, and this led on to the experimental proof of their antigenic action in the animal body, where they gave rise, on injection, to the production of the well-known "immune substances," such as bacteriolysins, complement-fixing bodies, precipitins and agglutinins.

Finally, a series of experiments was carried out by which it was established that these antigens can exert definite curative effects on animals suffering from bacterial infection.

In order to complete this section of the work, it was thought desirable to discover whether this somewhat heroic process of formalin-acetone extraction exercised a destructive effect on the essential antigen of Gram-negative bacteria, which might possibly prove to be more fragile than their Gram-positive brethren.

On subjecting *B. typhosus* to the process, it was shown that the extracted bacilli had retained their ability to cause the production of immune substances, such as specific precipitins and complement-fixing substances.

Neither in this case nor in any of the foregoing cases has it been established that none of the antigenic power is lost, but it is clearly proved that any loss that may occur can only be partial and therefore unimportant.

As a corollary of the main lines of research just described, a second and closely related train of thought suggested itself to me.

Pasteur's classical demonstration of the immunising properties of anthrax cultures "attenuated" by growth at 42°–43° C. has hitherto received nothing but the vaguest of explanations. Starting now from the new knowledge that "defatted" anthrax bacilli can be digested by trypsin, whereas the untreated bacilli are in the main indigestible, it seemed a reasonable hypothesis that growth at 42°–43° C. gives rise to a deficient formation of the normal "fatty" constituent, and thus renders the bacilli more easily susceptible to the digestive action of the body-fluids. Such bacilli, when injected into the living body, would be, at least in part, readily disintegrated, and the release of their antigen would cause a rapid immunity-response in the animal, before the Gram-positive, and therefore more resistant, bacilli developed in sufficient number to overwhelm the normal resistance of the body. In view of the foregoing considerations one was able to predict that growth at 42°–43° C. would be found to give rise to a weakening or disappearance of the Gram-positiveness of anthrax-bacilli. A few simple experiments proved this to be the case not only for that bacillus, but also for a stock laboratory strain of *Staphylococcus aureus*.

This latter organism was also shown to be digestible by trypsin after the formalin-acetone treatment, whereas Douglas had found it indigestible after acetone extraction alone.

The formalin-acetone process, whereby a relatively complete separation of "fatty" substances from the specific proteins is attained, has enabled me to throw light upon another set of unexplained phenomena.

It is well known that the subcutaneous injection of dead tubercle bacilli or staphylococci causes a severe local reaction with infiltration and sometimes necrosis, often apparently unaccompanied by any noteworthy general disturbance. In the light of our new knowledge the probability suggested itself that the local disturbance might be due entirely to the highly indigestible and irritating "fatty" constituents of the bacilli, and in no way to their specific antigenic proteins. This hypothesis was readily tested by the subcutaneous injection into suitable animals of the acetone extracts of tubercle bacilli and of staphylococci, previously treated with formalin, and the theory was shown to accord completely with the facts. The injection into normal animals of tubercle and staphylococcal "lipoids" gave rise to infiltrations which tended to persist for a long time; and in one case the undissolved mass of "lipoids" ulcerated through the skin and discharged itself, while a larger dose of defatted bacilli than corresponded to the dose of "lipoids" caused no local reaction in the same animal. We may take this as an additional proof that the "lipoids" of the bactericidal body form an important obstacle to the solvent and protective action of the body-fluids.

THE PROCEDURE FOR OBTAINING "DEFATTED" BACTERIAL ANTIGENS.

Definition of the Term "Defatted."

The term "defatted" is not intended to indicate that all the "lipoidal" substances present in the micro-organism have been removed. It merely implies that certain "lipoidal" substances, soluble in acetone after formalin-treatment, have been taken away.

In the case of the acid-fast and Gram-positive bacteria, the indication that the extraction has proceeded far enough for our purpose is the change of the staining reactions to non-acid-fastness or Gram-negativeness respectively.

Preparation of B. tuberculosis *Antigen.*

Tubercle bacilli are grown on the surface of glycerin-broth or other suitable liquid medium for a sufficient length of time, *e.g.* 2–3 weeks.

The fluid is decanted off and the mass of tubercle bacilli is transferred to an agate mortar. Some pure formalin can be added to the flask after the liquid has been removed in order to render the manipulations safer.

To the bacilli in the mortar a few drops of formalin (40 per cent. formaldehyde) are added, and the whole is ground up to a thick homogeneous paste. More formalin is then gradually added, with constant grinding, until 150–200 c.c. of formalin have been added to each 5 grm. of bacilli weighed wet.

The suspension of tubercle bacilli in formalin is now transferred to a flask fitted with a reflux condenser, and the flask is deeply immersed in a water-bath, which is heated to 100° C. and maintained at that temperature for 4 hours.

The suspension is now filtered through calcium-free filter-paper and the residue is washed three or four times with acetone until about 100° C. of acetone has been used.

The residue is next transferred to an extraction-thimble and this is placed in a Soxhlet apparatus with *ground-glass joints.* The whole is put in a water-bath in such a way that the extractor is partly immersed and the top of the thimble is approximately at the same level as the water in the bath. By this means the acetone around the thimble is kept at the boiling-point. The water-bath is kept at a temperature of 65°–70° C. and the extraction is allowed to proceed for 20–24 hours.

At the end of this time the insoluble residue is taken out, dried and ground in a sterile motar. If the bacilli are not then entirely non-acid-fast the process is repeated, *i. e.* the dried bacilli are again ground in formalin, heated for 4 hours at 100° C., washed with acetone, extracted with acetone for 20–24 hours at 65°–70° C., dried and ground up. It may be necessary to repeat the process a third, or even a fourth time, if the organisms are very resistant to extraction.

Preparation of suspension of T.B. antigen.—0·1 grm. of the dried extracted tubercle bacilli is weighed out with aseptic precautions and ground up very thoroughly in a sterile agate mortar.

Sterile saline solution (0·9 per cent.) is added drop by drop until, with continued grinding, a thick homogeneous paste is obtained. This is diluted gradually to 10·0 c.c. with the saline while grinding. It is then put in a sterile centrifuge-tube, which is closed with a sterile rubber stopper and centrifuged for 5–8 minutes at a speed of about 3000 revolutions per minute, until the milky supernatant fluid shows no granulation when examined with a six-magnification lens.

The supernatant fluid, when pipetted off and suitably diluted (see below) with sterile 0·9 per cent. saline containing 0·15 per cent. formalin, constitutes the antigen-suspension. The residue, together with a certain quantity of fluid at the bottom of the centrifuge-tube, must now be dried and weighed in order to estimate, by subtraction, how much of the original 0·1 grm. of "defatted" bacilli is contained in the antigen-suspension. Moreover, a slight correction is necessary in the calculation, for by drying the centrifuged deposit with a certain quantity of saline the weight of the salt in the saline has been added to that of the residual "defatted" bacilli.

The quantity of fluid left in the centrifuge tube is clearly equal to 10 c.c. minus the number of c.c. pipetted off (which must be measured), and the weight of NaCl in grammes is, of course, 0·9 per cent. of the quantity of fluid left in the tube.

Preparation of stock suspension.—Having thus ascertained the actual weight of "defatted" organisms in the suspension, the latter is diluted with sterile 0·9 per cent. saline, containing 0·15 per cent. formalin, in such a manner that *one cubic centimetre* of the fluid suspension contains *one fifth of a milligramme of dry "defatted" tubercle bacilli.*

Preparation of suspension for use.—These are made up from the stock suspension as required, and always contain a definite weight of "defatted" T.B. per volume of fluid. The quantities vary according to the purpose for which they are to be used—as, for instance, for treatment of infections; for injection into normal animals in the production of bacteriolysins, precipitins, complement-fixing substances and agglutinins, or for use as test-suspensions in complement-fixation or agglutination-experiments.

Preparation of "Defatted" Antigens of Bacteria other than B. tuberculosis.

By the process just described, "defatted" antigens of human and bovine tubercle bacilli, *B. anthracis*, staphylococcus, streptococcus, pneumococcus, *B. diphtheriæ*, *B. lepræ*, *B. typhosus*, gonococcus, etc., have been prepared. The method is doubtless applicable to all bacteria, whether acid-fast, Gram-positive or Gram-negative.

The ease and rapidity of extraction differ in different bacteria. For example, some strains of the streptococcus have been found very refractory. Again, a bacterium grown under varying conditions, as regards culture-medium and temperature, may vary greatly in the ease with which it can be extracted. But in all cases, by sufficient repetition of the process, it is possible to render the acid-fast and Gram-positive bacteria completely non-acid-fast or Gram-negative.

The Quantities of "Lipoidal" Substances that can be Removed from Various Bacteria.

Although the details of this subject still remain to be worked out, it is possible to state that the quantity of "lipoidal" substances obtainable with the formalin-acetone process from the tubercle bacillus is very much greater than that which can be removed from the non-acid-fast Gram-positive bacteria. The latter, in their turn, yield considerably more than the Gram-negative micro-organisms.

Again, the quantities obtainable from any given bacterium vary with the constitution of the medium on which it is grown, with the period of growth and the temperature of incubation.

The Microscopic Appearances of "Defatted" Antigen.

According to Salimbeni (1912) extraction of tubercle bacilli with trichlorhydrine rapidly makes them non-acid-fast and granular, and soon reduces them to an amorphous mass which does not take up the stain.

After extracting by the formalin-acetone process, the residue finally becomes completely non-acid-fast, and, on staining by ordinary methods, one finds that the bacillary character of the residue (*B. tuberculosis* antigen) has apparently been lost. The counterstain in Ziehl-Neelsen's method with ordinary aniline dyes merely shows faintly-stained amorphous masses. But, by means of prolonged staining with carbol-fuchsin, it is quite easy to demonstrate that the material really consists of bacilli, which stain too feebly to be demonstrated by the customary methods.

Technique.—A thin, uniform film of the antigen is made, dried, and fixed with ether-alcohol, followed by flaming.

An oblong piece of filter-paper is cut out so as to lie on the slide, covering the film, and extending nearly to the edges of the slide.

Carbol-fuchsin (Ziehl-Neelsen) is poured drop by drop on to the centre of the filter-paper, until this is thoroughly soaked all through. Air-bubbles between the filter-paper and the slide must be avoided. The slide is now warmed until steam rises, and then left to stain for 10 to 20 minutes.

Distilled water is then run on to the slide to wash off the stain and the filter-paper. The slide is then carefully washed until no more stain is given off. The film is then dried and examined in the usual way.

Demonstrated in this manner, the "defatted" tubercle bacilli appear as rods of extreme fineness and reduced length. Many of them are only faintly stained. Their average thickness is about that of the *Spirochæta pallida*, or even less. The leprosy bacillus undergoes a similar change. The degrees of reduction in size and of alteration in staining properties vary in the different groups of bacteria in proportion to the quantities of "lipoidal" substances that are removed. The non-acid-fast, Gram-positive bacteria undergo less profound alteration than the acid-fast group; they become Gram-negative, take the counterstain badly, and betray a noteworthy reduction in size. In the Gram-negative group the changes are the least marked of all, but even here are quite perceptible. The organisms become somewhat smaller and stain less intensely after extraction than before.

DIGESTIBILITY BY TRYPSIN OF "DEFATTED" BACTERIA.

It is generally accepted that proteins, after treatment with formaldehyde, cannot be digested with trypsin. Nevertheless all the "defatted" micro-organisms subjected to this test have proved to be digestible. Very dense suspensions can be almost entirely cleared with 1 per cent. trypsin (*injectio trypsini*, Fairchild).

The rate and completeness of the digestion, however, depend on a number of factors which remain at present undetermined, as, for instance, the amount of formalin retained in the proteins of the bacteria, the hydrogen-ion concentration, salt-content and so forth. It appears that very small alterations in these conditions can be responsible for very pronounced differences in the course of the reaction. One can, for instance, take the same "defatted" bacteria, grind them up in saline on two different occasions, add the same quantity of alkali and of trypsin, and maintain at the same temperature for the same length of time, and yet obtain, on the one occasion a perfect digestion, and on the other a relatively poor result, with only slight clarification.

These irregularities appear to be more pronounced with the tubercle-antigen than with the others, but they occur in all. At present, therefore, the preparation of a perfect digest is frequently a matter of considerable difficulty.

Preparation of Precipitinogens from "Defatted" Antigens.

The method used for the preparation of precipitinogen for the testing of a precipitating serum consists of the digestion of the "defatted" antigens with trypsin—a procedure based upon that employed by Sanarelli (1920), S. R. Douglas (1921) and others. Douglas showed that a precipitinogen resulted from the trypsin-cleavage of acetone-extracted Gram-negative bacteria.

Method.—The antigen-powder is ground up in sterile saline solution in an agate mortar to a dense suspension.

This is alkalised faintly, and from 1 to 2 per cent. of Fairchild's trypsin is added. Digestion at 37° C. is now allowed to proceed for a period of not less than 2 hours and not more than 24. A more precise statement of the optimum time for digestion or of other optimal conditions is not at present possible.

As soon as it is thought that the digestion has proceeded far enough, the pH of the fluid is tested and brought back to the point at which it started.

It is at present uncertain whether it is best to heat for 10 mins. to 70° C. to destroy the trypsin or to leave it unheated. The fluid is now either centrifugalised or filtered through a coarse porcelain filter, whereupon it is ready for use.

Variability of digests.—Just as different specimens of defatted antigen vary in digestibility, so different digests of the same specimen of antigen, in spite of apparently identical conditions of preparation, show considerable variations in their sensitiveness as regards their reaction with precipitating sera. One digest may be highly sensitive, and another almost completely inert.

In this respect the time of digestion undoubtedly exercises an important influence, for a prolonged digestion, *e.g.* 30 hours, decreases the value of the resulting precipitinogen. The importance of other less defined factors remains for investigation, such as different hydrogen-ion concentration, salt-content, and a possible variation in the strength of the trypsin in different ampoules, due to varying chemical composition of the glass.

EXPERIMENTS WITH "DEFATTED" ANTIGENS.

Under this heading will be described experiments with a considerable variety of "defatted" antigens, arranged under headings of the various bacteria from which the antigens were made. The first and most prominent position is given to *B. tuberculosis*, because it is in many ways the most important of all, and because it was in connection with this organism that the idea of obtaining an adequately "defatted" antigen first arose. For this reason a more thorough study has so far been made of the tubercle-antigen than of any other.

EXPERIMENTS WITH B. TUBERCULOSIS.

I. Production of Immune Substances by Injection of "Defatted" Antigen into Normal Rabbits.

The "defatted" T.B. antigen referred to here, and throughout the paper, was made with a strain of *B. tuberculosis* known as "Lyons." The antigen contained 0·2 mgrm. of "defatted" bacilli per cubic centimetre.

Four rabbits, I, II, III, and IV, received the treatment shown in Table I.

As a result of the injections of "defatted" T.B. antigen these rabbits developed in varying degrees the following anti-substances, the presence of which in the serum was demonstrated by test-tube experiments—complement-fixing bodies, precipitins and agglutinins. The details will be found in the records and discussion of the *in vitro* experiments on pp. 157–159.

TABLE I

Nº OF RABBIT	DATE	SEX	WEIGHT	DOSE OF DEFATTED ANTIGEN 1 CC = 0·2 mg	ROUTE	REMARKS
I	29·12·22	FEMALE	2200	0·5 cc	INTRAVENOUS	
	4·1·23		2270	1·0 cc	"	
	17·3·23		2230	1·0 cc	"	12·2·23 HAD YOUNG ALL DEAD
	27·3·23		2200	1·0 cc	"	
	4·4·23		2420	1·0 cc	"	
	12·4·23		2555	1·0 cc	"	
	19·4·23		2050	1·0 cc	"	
	29·4·23		2040	1·0 cc	"	16·4·23 HAD YOUNG LIVING
	18·5·23		2220	–	–	
II	29·12·22	FEMALE	2150	0·5 cc	INTRAVENOUS	
	4·1·23		1950	1·0 cc	"	
	17·3·23		2390	1·0 cc	"	
	27·3·23		2670	1·0 cc	"	
	4·4·23		2620	1·0 cc	"	
	12·4·23		2590	1·0 cc	"	
	19·4·23		2550	1·0 cc	"	
	29·4·23		2800	1·0 cc	"	
	18·5·23		3190	–	–	
III	31·12·22	MALE	2000	1·0 cc	SUBCUTANEOUS	NO INFILTRATION
	8·1·23		1970	1·0 cc	"	SMALL HÆMATOMA CAUSED BY INJECTION. GONE IN 2 DAYS
	17·3·23		2370	1·0 cc	"	NO INFILTRATION
	27·3·23		2340	1·0 cc	INTRAVENOUS	
	4·4·23		2340	1·0 cc	"	
	12·4·23		2390	1·0 cc	"	
	19·4·23		2350	1·0 cc	"	
	29·4·23		2370	1·0 cc	"	
	18·5·23		2470	–	–	
IV	31·12·22	MALE	2320	1·0 cc	SUBCUTANEOUS	NO INFILTRATION
	8·1·23		2320	1·0 cc	"	" "
	17·3·23		2350	1·0 cc	"	" "
	27·3·23		2410	1·0 cc	INTRAVENOUS	
	4·4·23		2500	1·0 cc	"	
	12·4·23		2520	1·0 cc	"	
	19·4·23		2500	1·0 cc	"	
	29·4·23		2620	1·0 cc	"	
	18·5·23		2600	–	–	

Technique of In Vitro *Tests.*

(A) *Complement-fixation.*

The experiments were carried out in dwarf test-tubes of about 1 cm. in diameter and 7 cm. in length, exactly similar to those employed as dilution-tubes in the agglutination technique.

Titration of complement.—Falling quantities of complement (*i.e.* normal guinea-pig serum) are added to fixed quantities of sensitised sheep's red cells and incubated for 30 minutes in the water-bath at 37° C. Thus:

Guinea-pig serum 1/20 .	10	9	8	7	6	5	4	3	2	1	0 drops.
0·9 per cent. saline solution	0	1	2	3	4	5	6	7	8	9	10 drops.
	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Sensitised sheep's red cells	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25 c.c.

N.B.—The saline is added to each tube in two stages—firstly, a sufficient number of drops to make serum *plus* saline always equal to 10 drops; secondly, 1 c.c. to each tube.

Titration of antigen.—Falling quantities of antigen are added to a fixed quantity of complement (2 minimal hæmolytic doses) and incubated for 1 hour in the water-bath at 37° C.; sensitised sheep cells are added, and the tubes are shaken and replaced in the bath for 1 hour.

The complement is diluted so that 5 drops contain 2 minimal hæmolytic doses. A control tube containing 15 drops antigen and no complement is included. Thus:

Complement	5	5	5	5	5	5	5	5	5	5 drops.
Antigen	15	12	10	8	6	5	4	3	2	1 „
Saline	0 1c.c.	3 1c.c.	5 1c.c.	7 1c.c.	9 1c.c.	10 1c.c.	11 1c.c.	12 1c.c.	13 1c.c.	14 „ 1c.c.
Sensitised sheep's red cells	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25 c.c.

After 1 hour's incubation sensitised red cells are added, and the whole is reincubated for 1 hour at 37° C.

Test.—Falling quantities of the serum to be tested are added to fixed quantities of antigen and complement (2 M.H.D.) and incubated for 1 hour at 37° C.

Sensitised sheep cells are added. The tubes are shaken and replaced in the bath for 1 hour.

The antigen is diluted so that the largest dose which is not anti-complementary is contained in 15 drops.

The complement is diluted so that 2 hæmolytic doses are contained in 5 drops.

A control-tube to exclude anti-complementary action on the part of the test serum is included. Thus:

Tube	C (control)	1	2	3	4	5	6	7 etc.
Serum to be tested	5	5 (1:1)	2	1	10 (1:20)	5	2	1 drops.
Saline	20 1c.c.	5 1c.c.	8 1c.c.	9 1c.c.	0 1c.c.	5 1c.c.	8 1c.c.	9 „ 1c.c.
Complement (2 M.H.D. per tube)	5	5	5	5	5	5	5	5 drops.
Antigen	0	15	15	15	15	15	15	15 „

The tubes are incubated for 1 hour at 37° C., after which 0·25 c.c. of sensitised sheep's red cells is added to each tube.

The tubes are shaken and replaced in the bath for 1 hour.

(B) *Precipitin reaction.*

This is carried out in the small pointed tubes known as Dreyer's agglutination tubes, either by adding 5 drops of the serum to each tube, and then 15 drops of a series of previously prepared graded dilution of antigen-digest, each dilution being double the previous one; or by adding falling quantities of serum to 15 drops of antigen-digest suitably diluted.

The results are read after incubation at 56° C. for 6 and 20 hours respectively.

(C) *Agglutination tests.*

These were carried out with the technique known as Dreyer's method, which is described in the Directions sent out by the Standards Laboratory, the University, Oxford, on behalf of the Medical Research Council.

II.—Production of Immune Substances in already Infected Rabbits with or without Injection of "Defatted" T.B. Antigen.

Rabbits V and VI were injected subcutaneously in the middle of the

TABLE II

RABBIT V.; MALE, INJECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. (SEE TEXT). AT THE TIME OF THE FIRST INJECTION OF DEFATTED T.B. ANTIGEN THERE WAS A NODULE 2·0 cm IN DIAMETER AT THE SITE OF INFECTION.

DATE	WEIGHT	DOSE OF DEFATTED ANTIGEN	ROUTE	REMARKS
12.1.23	2380	—	—	
18.2.23	2570	0·5 cc	SUBCUTANEOUS	24 HOURS AFTER THIS INJECTION A WELL MARKED HARDISH INFILTRATION APPEARED WHICH GRADUALLY DIMINISHED, AND HAD DISAPPEARED ON THE 5TH DAY.
8.3.23	2600	0·5 cc	INTRAVENOUS	
18.3.23	2390	1·0 cc	"	
28.3.23	2390	0·5 cc	"	
4.4.23	2600	0·5 cc	"	
12.4.23	2500	0·5 cc	"	
19.4.23	2400	0·5 cc	"	
29.4.23	2720	0·75 cc	"	
18.5.23	2820	—	—	

TABLE III

RABBIT VI – FEMALE, INJECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. ON 15.12.23 IT HAD A NODULE ABOUT 1·5 cm IN DIAMETER AT SITE OF INFECTION.

DATE	WEIGHT	DOSE	ROUTE	REMARKS
12.1.23	2400	–	–	
15.2.23	2690	0·5 cc	SUBCUTANEOUS	MARKED INFILTRATION AT SITE OF INJECTION AFTER 24 HOURS, WHICH SLOWLY DECREASED BUT DID NOT ENTIRELY DISAPPEAR TILL THE 10TH DAY
8.3.23	2800	0·5 cc	INTRAVENOUS	
17.3.23	2600	1·0 cc	"	
27.3.23	2800	0·5 cc	"	
4.4.23	2750	0·5 cc	"	HAD LITTER OF YOUNG ON 3.4.23.
12.4.23	2850	0·5 cc	"	
19.4.23	2850	0·5 cc	"	
29.4.23	2890	0·75 cc	"	
18.5.23	3030	–	–	

abdomen on 12 . i . 23 with 0·5 c.c. of a suspension of a strain of a living bovine tubercle bacillus (Pasteur Institute), here referred to as "A." The suspension contained about 200 to 300 million bacilli per c.c.

TABLE IV.

RABBIT VII – FEMALE, INFECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. (SEE TEXT). AT TIME OF FIRST ANTIGEN INJECTION THERE WAS A NODULE ABOUT 1·5 cm IN DIAMETER AT SITE OF INFECTION.

DATE	WEIGHT	DOSE	ROUTE	REMARKS
12.1.23	2200	–	–	
15.2.23	2540	0·5 cc	SUBCUTANEOUS	LARGE SWELLING WITH HYPERÆMIA AFTER 24 HOURS DECREASED GRADUALLY, DISAPPEARING ENTIRELY ON THE 7TH DAY.
8.3.23	3050	0·5 cc	INTRAVENOUS	
17.3.23	2450	1·0 cc	"	
28.3.23	2550	0·5 cc	"	
4.4.23	2840	0·5 cc	"	
12.4.23	2940	0·5 cc	"	HAD YOUNG. 13.4.23.
19.4.23	2700	0·5 cc	"	
29.4.23	3050	0·75 cc	"	
18.5.23	2920	–	–	HAD YOUNG. ALL DEAD.

TABLE V.

RABBIT VIII – MALE, INFECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. ON 15.2.23 THERE WAS A NODULE OF ABOUT 2·0 cm IN DIAMETER AT SITE OF INFECTION BREAKING THROUGH SKIN, AND A GLAND IN THE RIGHT AXILLA THE SIZE OF A FILBERT NUT.

DATE	WEIGHT	DOSE	ROUTE	REMARKS
12.1.23	2600	–	–	–
15.2.23	2850	–	–	–
8.3.23	3070	–	–	NODULE OPEN, ULCERATING.
17.3.23	3040	–	–	–
27.3.23	2850	–	–	–
4.4.23	2900	–	–	LARGE AXILLARY GLAND.
12.4.23	2840	–	–	–
19.4.23	2800	–	–	ULCERATION OF ABOUT 2·5 cm OVER NODULE
29.4.23	2980	–	–	–
18.5.23	2540	–	–	ULCER UNCHANGED; GLAND SLIGHTLY LARGER

Rabbits VII and VIII received on 12 . i . 23 a subcutaneous abdominal injection of 0·5 c.c. of a suspension of another strain of a living bovine tubercle bacillus (Pasteur Institute) here referred to as "B." This suspension was thinner, containing only about 100–200 million bacilli per c.c.

The production of immune substances in these animals and in the normal animals previously referred to was as follows:

III.—The Character of the Immune Substances Developed.

Complement-fixation Tests.

From Table VI it is clear that so far as complement-fixation is concerned the "defatted" T.B. antigen *is specific* in its reaction, not only with the serum derived from a horse immunised with whole tubercle bacilli, but also with the serum of a rabbit suffering from tuberculosis.

As *controls* of the test of the anti-tuberculous horse-serum, strong anti-diphtheritic sera were used, in order to ascertain whether the serum of an animal which had developed a high immunity to a totally different antigen gave any reaction with the "defatted" tubercle bacilli. This is clearly not the case.

TABLE VI

DESCRIPTION OF SERUM	QUANTITY OF SERUM IN DROPS*	RELATIVE STRENGTH OF SERA
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/10	500
ANTIDIPHTHERITIC HORSE-SERUM-400 UNITS	>5	<1·0
ANTIDIPHTHERITIC HORSE-SERUM-500 UNITS	5	1·0
RABBIT INJECTED WITH LIVING BOVINE B. TUBERCULOSIS	1/2	10·0
NORMAL RABBIT 1.	5	1·0
NORMAL RABBIT 2.	5	1·0

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GAVE COMPLETE COMPLEMENT-FIXATION.

The "defatted" antigen having proved to be specific, the next step was to investigate the complement-fixation reactions of normal animals injected with "defatted" T.B. antigen, and of rabbits suffering from tuberculosis, some of which had been treated with "defatted" antigen, while others had not.

Table VII shows that the injection of "defatted" antigen into normal rabbits gives rise to complement-fixing substances. On the whole, the infected animals, subsequently injected with "defatted" antigen, seemed to give stronger reactions than either those that were simply infected, or the normals that had received "defatted" antigen. But it is impossible to draw a definite conclusion on this point from such a small number of experiments.

TABLE VII

DESCRIPTION OF SERUM		QUANTITY OF SERUM IN DROPS *	RELATIVE STRENGTH OF SERA
NORMAL RABBITS INJECTED WITH DEFATTED T.B. ANTIGEN	Nº I	1	5·0
	II	2	2·5
	III	2	2·5
	IV	3	1·7
RABBITS INFECTED WITH LIVING T.B. AND SUBSEQUENTLY INJECTED WITH DEFATTED T.B. ANTIGEN	V	1	5·0
	VI	1/4	20·0
	VII	1/4	20·0
RABBIT INJECTED WITH LIVING T.B. NO SUBSEQUENT TREATMENT	VIII	1	5·0
NORMAL RABBITS	A	>5	<1·0
	B	>5	<1·0

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GAVE COMPLETE COMPLEMENT-FIXATION.

It was furthermore of interest to compare the activity of the "defatted"

TABLE VIII

COMPARISON BETWEEN DEFATTED T.B. ANTIGEN AND THE ANTIGEN OF BOQUET & NÈGRE

DESCRIPTION OF SERA		DEFATTED T.B. ANTIGEN		ANTIGEN OF BOQUET & NÈGRE	
		QUANTITY OF SERUM IN DROPS *	RELATIVE STRENGTH OF SERA	QUANTITY OF SERUM IN DROPS *	RELATIVE STRENGTH OF SERUM
NORMAL RABBITS INJECTED WITH DEFATTED T.B. ANTIGEN	Nº I	½	10·0	1	5·0
	II	½	10·0	7	0·7
	III	2	2·5	8	0·6
	IV	5	1·0	6	0·8
RABBITS INFECTED WITH LIVING T.B. AND SUBSEQUENTLY INJECTED WITH DEFATTED T.B. ANTIGEN	V	1	5·0	6	0 8
	VI	¼	20·0	7	0 7
	VII	½	10·0	2	2·5
RABBIT INJECTED WITH LIVING T.B. NO SUBSEQUENT TREATMENT	VIII	1	5 0	1	5·0
NORMAL RABBITS	A	7	0·7	7	0·7
	B	8	0·6	8	0·6

* I.E THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GIVES COMPLETE COMPLEMENT-FIXATION.

antigen with that of a recognised antigen of the usual type, and for this purpose Boquet's and Nègre's antigen was chosen. From Table VIII it is evident that the "defatted" antigen in the majority of cases shows by far the greater activity of the two.

TABLE IX

FALLING QUANTITIES OF SERUM. FIXED QUANTITY OF DIGEST OF DEFATTED ANTIGEN.

DESCRIPTION OF SERUM		HIGHEST DILUTIONS OF SERUM SHOWING EQUAL DEGREES OF PRECIPITATION	RELATIVE ACTIVITY OF SERA
RABBITS INFECTED WITH LIVING T.B. AND SUBSEQUENTLY INJECTED WITH DEFATTED ANTIGEN	Nº V	1/25	10·0
	VI	1/12·5	5·0
	VII	1/25	10·0
RABBIT INFECTED WITH LIVING T.B. NO OTHER TREATMENT	VIII	1/50	20·0
NORMAL RABBITS	A	>1/2·5	<1·0
	B	>1/2·5	<1·0

TABLE X

FIXED QUANTITY OF SERUM. GRADED DILUTIONS OF DIGEST OF DEFATTED ANTIGEN.

		HIGHEST DILUTIONS OF PRECIPITINOGEN SHOWING EQUAL DEGREES OF PRECIPITATION	RELATIVE ACTIVITY OF SERA
RABBITS INJECTED WITH DEFATTED T.B. ANTIGEN	Nº I	1/170	5·3
	II	1/42	1·3
	III	1/64	2·0
	IV	1/128	4·0
RABBITS INFECTED WITH LIVING T.B. THEN INJECTED WITH DEFATTED T.B. ANTIGEN	V	1/256	8·0
	VI	1/128	4·0
	VII	1/128	4·0
RABBIT INFECTED WITH LIVING T.B. NO OTHER TREATMENT	VIII	1/128	4·0
NORMAL RABBITS	A	1/32	1·0
	B	1/32	1·0

Precipitin Reactions.

These are shown in Tables IX and X.

In Table IX it is seen that the T.B. precipitinogen obtained by digesting "defatted" T.B. with trypsin is *specific* in its reaction. This corresponds with S. R. Douglas's experience in the case of acetone-extracted Gram-negative bacteria. The sera of animals suffering from tuberculosis, whether injected with defatted antigen or not so treated, give precipitation with the digest. That the injection of defatted T.B. antigen into normal rabbits gives rise to specific precipitins is shown in Table X. As in the case of complement-fixation, there is here also an indication that the highest reactions are given by animals which have been infected with the tubercle bacillus, and subsequently treated with "defatted" antigen.

Agglutination Experiments.

Tables XI and XII illustrate the agglutination experiments, which were performed with saline suspensions of the "defatted" bacilli diluted to a suitable constant opacity with normal saline formolised to 0·1 per cent.

Table XI shows that the sera of tuberculous animals contain agglutinins which react *specifically* with the "defatted" bacilli. From Table XII we can conclude that the injection of "defatted" antigen into normal rabbits causes the production of specific agglutinins, and that these substances are also present in the serum of animals infected with the tubercle bacillus whether they have or have not received subsequent injections of "defatted" antigen. Once more the combination of infection with injection seems to yield, on an average, the strongest sera.

TABLE XI

DESCRIPTION OF SERA	HIGHEST DILUTIONS OF SERA SHOWING EQUAL DEGREES OF AGGLUTINATION	RELATIVE ACTIVITY OF SERA
HORSE IMMUNIZED WITH TUBERCLE BACILLUS	1/100	40·0
HORSE ANTI-ANTHRAX SERUM (MULFORD)	> 1/2·5	< 1·0
TUBERCULOUS RABBIT	1/12·5	5·0
NORMAL RABBIT	>1/2·5	< 1·0

TABLE XII

DESCRIPTION OF SERA		HIGHEST DILUTIONS OF SERA SHOWING EQUAL DEGREES OF AGGLUTINATION	RELATIVE ACTIVITY OF SERA
NORMAL RABBITS INJECTED WITH DEFATTED T. B. ANTIGEN	Nº I	1/250	100·0
	II	1/250	100·0
	III	1/50	20·0
	IV	1/25	10·0
RABBITS INFECTED WITH LIVING T. B. AND SUBSEQUENTLY INJECTED WITH DEFATTED T. B. ANTIGEN	V	1/100	40·0
	VI	1/500	200·0
	VII	1/250	100·0
RABBIT INJECTED WITH LIVING T. B. NO SUBSEQUENT TREATMENT	VIII	1/100	40·0
RABBIT INJECTED WITH FATTY EXTRACT OF T.B. EMULSIFIED IN SALINE	23	>1/2·5	<1·0
NORMAL RABBITS	X	1/5	2·0
	Y	1/2·5	1·0

IV. Treatment with "Defatted" T.B. Antigen of Animals Infected with *B. tuberculosis.*

The experiments detailed in this section were performed with a view to studying the therapeutic effects of "defatted" tubercle-antigen on animals suffering from well-defined tuberculosis.

Guinea-pigs.

Four animals have been thoroughly studied, *i. e.* Nos. 4, 26, 27 and 28.

Nos. 26, 27 and 28 were kindly given to me by Capt. S. R. Douglas, with the object of accelerating my results. Nos. 27 and 28 had been infected with *B. tuberculosis* by Capt. Douglas on April 20th, 1922. No. 26 had been similarly infected, but neither the exact date of infection nor the weight of the animal at the time can be traced. The infection, however, took place several months before treatment was begun. Guinea-pig 4 was infected in my laboratory on June 15th, 1922, with the same strain of tubercle bacillus as had been used by Douglas.

The bacillus used, "T.B. 100," is the strain used at the Höchst laboratory for making tuberculin. Douglas received it from Schottelius in 1908. It is a bacillus of the human type, and has never passed through an animal since it has been in Douglas's possession. He has injected at least 50 guinea-pigs with this strain during the last two years. The culture is, on the whole, of slight virulence. On good media it grows well, and definite growth may be obtained in 3 to 4 days. The infected animals have usually died in 4 to 10 months (most commonly 6 to 8 months) after infection, and the *post-mortem* examination has invariably shown a generalised tuberculosis, with the following features: *Spleen* enlarged, with sago granules, but no large areas of disease. *Liver* somewhat enlarged, with similar granules. *Lungs* showing pinhead tubercles. *Lymph-glands*: the axillary and cervical glands showed well-marked enlargement (size of a pea or more); the inguinal and lumbar glands always enlarged and caseous.

In smears of the spleen and lymph-glands the tubercle bacillus could always readily be demonstrated in considerable numbers, and cultures were easily obtained.

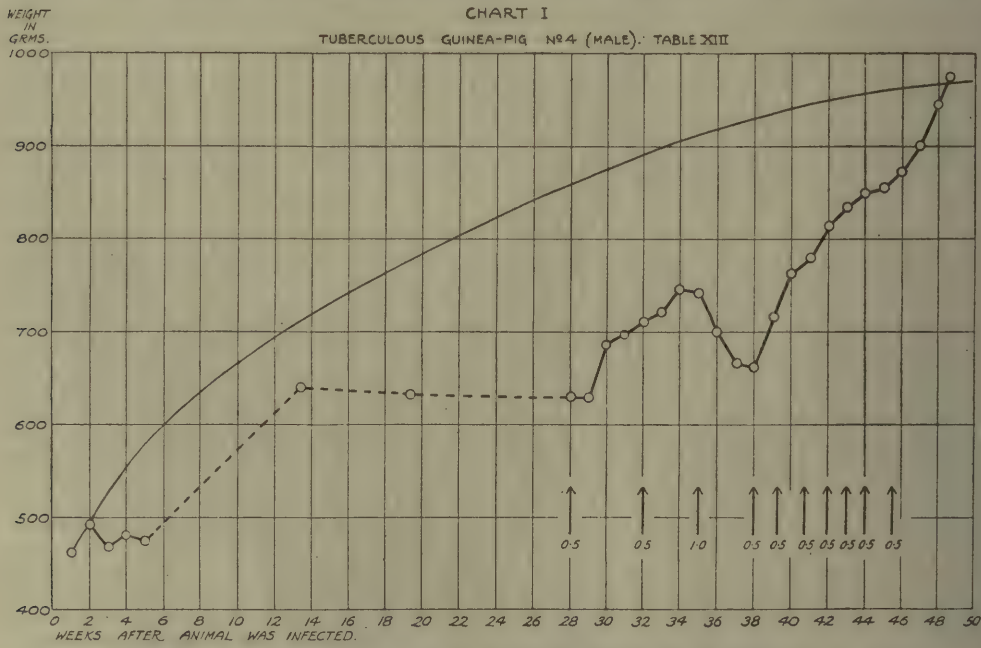
The animals 26, 27 and 28 were the last survivors of a batch of 25 that were injected on April 20th, 1922. At the time of injection most of them weighed between 400 and 500 grm. Many of this batch were healthy survivors from the testing of diphtheria antitoxin.

At the time when treatment was started all animals showed well-marked signs of tuberculosis. The details of the condition of the animal and of the injections will be found under each animal.

All the animals were weighed every day from the beginning of treatment, but the weights given in the tables are averages of 7 days' weighing.

Guinea-pig No. 4 (male). (See Table XIII and Chart I.)

Condition before treatment.—Treatment was begun on 29 . xii . 22, 28 weeks after the animal had been infected with T.B. 100. The animal looked



N.B. A CURVE OF THE NORMAL PROGRESS IN WEIGHT OF ANIMALS OF THE SAME AGE AS G.P. 4, CALCULATED FROM 6 HEALTHY ANIMALS, IS INCLUDED FOR COMPARISON.

TABLE XIII.

TUBERCULOUS GUINEA-PIG Nº 4 (MALE).

DATE	AVERAGE WEEKLY WEIGHT *	WEEKS FROM INFECTION	INJECTIONS DATE & DOSE	REMARKS
21.6.22	462	1		
28.6.22	490	2		
5.7.22	468	3		
12.7.22	480	4		
19.7.22	475	5		
16.9.22	640	13 3/7		
				21.10.22 TESTED WITH OLD TUBERCULIN GRADED VON PIRQUET STRONGLY POSITIVE.
28.10.22	633	19 3/7		
28.12.22	630	28		
			29.12.22 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 10 DAYS.
4.1.23	629	29		
11.1.23	685	30		
18.1.23	696	31		
			24.1.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 6 DAYS.
25.1.23	710	32		
1.2.23	720	33		
8.2.23	746	34		
15.2.23	740	35	15.2.23 1·0cc	INJECTION FOLL. BY LARGE INFILTRATION LASTING 13 DAYS.
22.2.23	700	36		
1.3.23	666	37		
8.3.23	661	38	8.3.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 3 DAYS.
15.3.23	716	39		
			17.3.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 7 DAYS.
22.3.23	763	40		
			27.3.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
29.3.23	780	41		
			4.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
5.4.23	815	42		
12.4.23	836	43	12.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 4 DAYS.
19.4.23	850	44	19.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 5 DAYS.
26.4.23	856	45		
			29.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 4 DAYS.
3.5.23	874	46		
10.5.23	906	47		
17.5.23	946	48		

* I.E. AVERAGE OF 7 DAYS WEIGHING, ENDING ON DATE GIVEN.

healthy, but was under the average weight for its age, scaling 630 grm. instead of 800 to 900 grm. The spleen was large and could easily be felt. There were 3 or 4 enlarged glands in the right inguinal region, and 1 or 2 on the left side. Those on the right were about the size of a large pea, those on the left the size of a small pea. There was diffuse infiltration around the glands.

Condition after treatment (48 weeks after infection).—The animal looks perfectly healthy, and is now of the normal weight for its age. The spleen seems smaller. The inguinal glands on the right side are smaller and harder. The glands on the left side have practically disappeared, being replaced by what feels like a pad of fibrous tissue.

In an animal of this species the weight is the best indication of the general condition of health (see Table XIII and Chart I). In the absence of treatment this animal would have been expected to die within 10 months from the date of infection.

Guinea-pig No. 26 (male). (See Table XIV and Chart II.)

Condition before treatment.—The animal looked healthy. The spleen was large and could easily be felt. There were large masses of glands in the right and left inguinal regions, most marked on the right. About 2 months before treatment began the animal gave a positive von Pirquet reaction (graded).

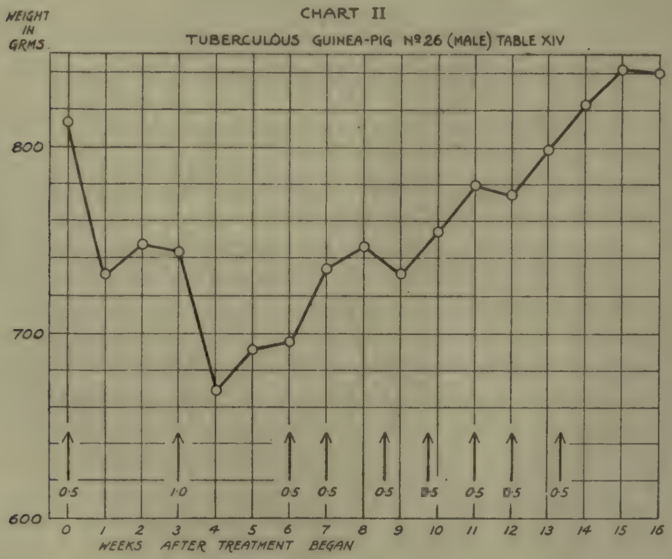
TABLE XIV

TUBERCULOUS GUINEA-PIG No 26 (MALE)

DATE	AVERAGE WEEKLY WEIGHT	WEEKS AFTER COMMENCEMENT OF TREATMENT	INJECTIONS DATE & DOSE	REMARKS
26.1.23	813	0	24.1.23 0·5cc	INJECTION FOLL. BY A VERY WELL-MARKED INFILTRATION LASTING 11 DAYS.
2.2.23	731	1		
9.2.23	747	2		
			15.2.23 1·0cc	INJECTION FOLL. BY A VERY LARGE INFILTRATION LASTING 14 DAYS.
16.2.23	743	3		
23.2.23	669	4		
2.3.23	691	5		
			8.3.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 3 DAYS.
9.3.23	695	6		
16.3.23	736	7		
			17.3.23 0·5cc	INJECTION FOLL. BY SMALL INFILTRATION LASTING 4 DAYS.
23.3.23	746	8		
			27.3.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
30.3.23	731	9		
			4.4.23 0·5cc	INJECTION FOLL. BY NO INFILTRATION.
6.4.23	754	10		
13.4.23	779	11	13.4.23 0·5cc	INJECTION FOLL. BY NO INFILTRATION.
			19.4.23 0·5cc	INJECTION FOLL. BY VERY SLIGHT INFILTRATION LASTING 4 DAYS.
20.4.23	774	12		
27.4.23	799	13		
			29.4.23 0·5cc	INJECTION FOLL. BY ?INFILTRATION LASTING 2 DAYS.
4.5.23	823	14		
11.5.23	842	15		
18.5.23	840	16		

CHART II

TUBERCULOUS GUINEA-PIG No 26 (MALE) TABLE XIV



Condition after treatment.—The animal looked healthy. Its weight is shown in Chart II. The glands in both inguinal regions had already entirely disappeared by 23 . iii . 23, *i.e.* 8 weeks after treatment began. On 18 . v . 23 no sign of any glands remained, though there was perhaps a slight residual thickening of the subcutaneous tissue.

Guinea-pig 28 (male). (See Table XV and Chart III.)

Condition before treatment.—Treatment was begun on 24 . i . 23, 39 weeks after infection with T.B. 100. The animal looked thin and poorly. It was greatly under weight, scaling only 540 grm. instead of 800–900 grm., which would have been the average normal weight of an animal of that age. The spleen was large and easily felt.

TABLE XV.

TUBERCULOUS GUINEA-PIG Nº 28 (MALE).

DATE	AVERAGE WEEKLY WEIGHT	WEEKS AFTER INFECTION	INJECTIONS DATE & DOSE	REMARKS
26.1.23	542	39	24.1.23 0·5 cc	INJECTION FOLL. BY MARKED INFILTRATION LASTING 5 DAYS.
2.2.23	508	40		
9.2.23	513	41		ANIMAL ILL, WITH PURULENT SECRETION FROM NOSE, LASTING TILL 28.2.23.
			15.1.23 1·0 cc	ANIMAL ILL, WITH LARGE INFILTRATION LASTING 5 DAYS.
16.2.23	514	42		
23.2.23	506	43		
2.3.23	503	44		ANIMAL HAS RECOVERED FROM ITS INTERCURRENT ILLNESS.
			8.3.23 0·5 cc	INJECTION FOLL. BY INFILTRATION LASTING 2 DAYS.
9.3.23	524	45		
16.3.23	572	46	17.3.23 0·5 cc	INJECTION FOLL. BY LARGE INFILTRATION LASTING 3 DAYS.
23.3.23	599	47		
30.3.23	578	48	27.3.23 0·5 cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
			4.4.23 0·5 cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 5 DAYS. ANIMAL ILL AGAIN WITH PURULENT SECRETION FROM NOSE, WHICH PERSISTED IN DIMINISHING DEGREE TILL 18.5.23.
6.4.23	595	49		
			12.4.23 0·5 cc	INJECTION FOLL. BY MARKED INFILTRATION LASTING 6 DAYS.
13.4.23	549	50		
20.4.23	556	51	20.4.23 0·5 cc	INJECTION FOLL. BY MARKED INFILTRATION LASTING 7 DAYS.
27.4.23	567	52		
			29.4.23 1·0 cc	INJECTION FOLL. BY LARGE INFILTRATION LASTING 8 DAYS. ANIMAL LOOKED THIN AND ILL, AND HAD DIARRHŒA WHICH WAS STILL PRESENT, THOUGH MUCH LESS ON 15.5.23. A SHARP DROP OF WEIGHT STARTED ON 52ND WEEK.
4.5.23	494	53		
11.5.23	525	54		
18.5.23	508	55		THE ANIMAL LOOKS MUCH BETTER, BUT STILL HAS SOME DIARRHŒA.

There were four or five large lymph-glands in right inguinal region and a similar number of still larger ones on the left side. One gland had ulcerated through the skin some 4 weeks before treatment. The ulcer was unhealthy looking and discharged caseous matter.

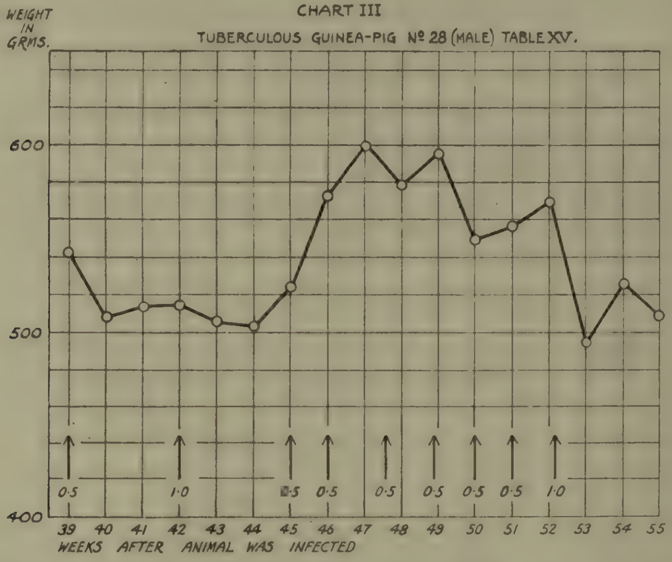
Condition during treatment.—Four days after the 1st injection the ulcer appeared smaller and clean. The discharge had ceased.

Ten days after the 1st injection the ulcer had healed up, and showed a nice smooth scar.

Within 24 hours of the 2nd injection the scar broke down, showing a healthy raw surface about 1 cm. long, without any discharge. It healed again within a week of the injection. Meanwhile the glands on both sides had definitely diminished in size.

CHART III

TUBERCULOUS GUINEA-PIG No. 28 (MALE) TABLE XV.



After the 3rd injection the scar again opened in about 66 hours. It was fully healed again in 6 days.

Twenty-four hours after the 4th injection the same thing occurred, and healing was complete in another 5 days.

After the 5th injection the scar remained healed. After the 6th it broke down slightly, to heal again in 3 days.

The 7th injection caused a tiny opening of the scar, which persisted 4 days.

On the 8th injection no breaking down occurred. Meanwhile a steady decrease in the size of the glands was in progress.

After the 9th injection the scar held firm.

Condition on 18 . v . 23.—The glands are greatly reduced in size. The ulcer is replaced by strong, healthy scar-tissue. The animal weighs a little over 500 grm. (see Chart III).

Guinea-pig No. 27 (female). (See Table XVI.)

Condition before treatment.—Treatment began on 24 . i . 23, 39 weeks after infection. The animal looked poorly. It was greatly under weight (550 grm. instead of a normal weight of about 800 grm.). The spleen was large and easily palpable. There was a mass of inguinal glands with diffuse periadenitis, which obscured the individual glands. No ulceration.

Condition during treatment.—The animal seemed to improve in appearance and the glands gradually diminished in size, becoming hard and discrete. The periadenitis had largely disappeared.

After the birth of its young the animal suddenly started to go downhill and died in a few days. It is impossible to state the cause of death with certainty. The heart-blood, cultivated in broth and on agar, remained sterile.

TABLE XVI

TUBERCULOUS GUINEA-PIG No. 27 (FEMALE)

DATE	AVERAGE WEEKLY WEIGHT	WEEKS FROM INFECTION	INJECTIONS DATE & DOSE	REMARKS
26.1.23	553	39	24.1.23 0·5 cc	MARKED INFILTRATION LASTING 11 DAYS.
2.2.23	540	40		
9.2.23	573	41		
			15.2.23 1·0 cc	LARGE INFILTRATION LASTING 9 DAYS.
16.2.23	567	42		
23.2.23	547	43		
2.3.23	543	44		
6.3.23				NOTICED TO BE PREGNANT
			8.3.23 0·5 cc	INFILTRATION LASTING 3 DAYS.
9.3.23	569	45		
16.3.23	649	46		
			17.3.23 0·5 cc	LARGE INFILTRATION LASTING 5 DAYS.
23.3.23	709	47		
			27.3.23 0·5 cc	SLIGHT INFILTRATION LASTING 3 DAYS.
30.3.23	717	48		
			4.4.23 0·5 cc	TRACE OF INFILTRATION LASTING 1 DAY.
6.4.23	726	49		
7.4.23				HAD YOUNG. 2 DEAD, 1 LIVING.
8.4.23				ANIMAL SUDDENLY ILL. LOST 100 grms. IN NEXT 3 DAYS.
11.4.23	438	(50)		
12.4.23				FOUND DEAD.

Since this animal was a female, and became pregnant, a graph of the weight would be of no interest.

Dr. A. G. Gibson kindly undertook to examine the organs of this guinea-pig, and reports as follows :

Guinea-pig No. 27 (female) ; *naked-eye appearances.*

The *inguinal glands* are enlarged to about the size of a lentil, with surrounding fibrosis, which is reddish. There is no caseation seen on section.

The *axillary glands* are enlarged ; one on the left side is the size of a pea.

The *spleen* is much enlarged, 5 by 2·5 cm. The surface is pale and anæmic with areas of congestion of irregular outline. These areas probably constitute what remains of normal spleen. Under the capsule can be seen dots suggesting miliary tubercles. On section the congested and anæmic areas are seen as on the surface. The pulp is firm, and the white areas suggest the consistency of a pale infarct. Neither the Malpighian bodies nor the trabeculæ can be distinguished.

The *liver* is diminished in size. The surface is irregular, especially that of the middle and left lobes. Three types of structure can be discerned :

1. Yellowish or greenish-yellow areas 3-4 mm. across, suggesting abscesses.

2. Anæmic areas suggesting granulomata, varying in size from that of a small pin's head to an area 6–8 mm. across.

3. Linear and radiate depressions—the result of shrinkage of liver-tissue. The rest of the liver shows congestion and fibrosis and in parts there is the appearance of hæmorrhage. The capsule is not thickened. On section the same three lesions are evident. The first contains semi-solid greenish necrotic substance. The second is pale, and suggests recently-formed fibrous tissue. The third suggests scarring as in human disease from the healing of an infarct or gumma.

Externally the *lungs* are congested, but show no evidence of gross disease, either externally or on section.

The *supra-renals* and *kidneys* are normal.

The *peritoneum*, both the *pleuræ* and the *pericardium*, show no lesions.

Microscopic Appearances.

Gland 1.—The section stained with hæmatoxylin and eosin shows two lymph-glands embedded in fatty tissue, with hyperæmia and some increase of cells. The gland itself shows both large and small areas unstained except for a variable number of nuclei. The gland-tissue has almost disappeared except at one end, and here and there in other parts. The pale area shows the ghosts of cells and nuclei, and in the centre of one there is a badly-stained giant-cell. The appearance of these ghost-like areas suggests healing tubercles that have subsequently undergone anæmic necrosis. The cells are of the endothelioid type, widely spaced from one another. Stained by van Gieson (Hansen's modification) these areas show abundance of collagen fibres giving them a redder appearance than the neighbouring parts of the gland. Practically the whole of the gland-tissue shows diffuse fibrosis, which is especially intense near the capsule. The only parts free from this change are the remains of the normal gland.

There is no evidence of necrosis or of caseation as is usually seen in tuberculous glands. Some pigmentation is present.

Gland 2.—Agrees in general features with No. 1, except that the pigmentation is more marked. The pale areas that lack full nuclear staining again show definite fibrosis. There is no evidence of necrosis or caseation, except for the appearances described. In the more cellular zone outside the pale areas there are fragments of nuclei but nothing that could be taken for necrosis, for side by side with these fragments there are complete and well-stained nuclei.

The *liver*, stained with hæmatoxylin and eosin, shows in every section granulomata, some highly cellular, and others less so. The first type shows fragments of nuclei, endothelioid cells, lymphocytes, and a few ill-formed giant-cells, but no evidence of necrosis or caseation. In the less cellular there is an eosin-staining matrix, in which can be made out a cellular structure suggesting liver-tissue. Here and there are nuclei, some ill-formed and irregular; some of these appear to be the lining cells of a capillary. These areas suggest infarction. The general lymphocytic reaction is small, except here and there, and some of the areas are full of fibroblasts with poorly-stainly nuclei. The liver-tissue elsewhere shows large fat-globules through the lobules. In Glisson's capsule there is seen proliferation of the bile-ducts. This is very marked in one section. Van Gieson's stain (Hansen's modification) shows a marked increase of fibrous tissue everywhere, but particularly of Glisson's capsule. The granulomata for the most part show a peripheral zone of fibrous tissue, which in some cases seems to be spreading inwards. Fibrous tissue is not present in the area that shows infarction. There is much congestion of the liver-tissue in parts. A marked increase in the number of bile-ducts is seen in one part.

The *lungs*, stained with *hæmatoxylin and eosin*: Abnormal areas are present in the lungs, involving the connective tissue accompanying the artery and bronchus, and invading the lung-tissue in the neighbourhood. The alveoli here are largely obliterated, the septa are thickened and the alveoli and bronchioles contain catarrhal cells. Only here and there is there some round-celled lymphocytic infiltration. The *van Gieson-stained* section shows in these areas early general fibrosis, most intense in the peri-arterial tissues. Fibrous tissue is penetrating the whole thickness of the bronchial wall. Elsewhere the lung shows slight catarrh and congestion.

The *spleen*, stained with hæmatoxylin and eosin: The large pale areas appear as patches of anæmic necrosis, the cellular structure being ill-defined by reason of faulty staining of nuclei. Careful examination, however, reveals a granulomatous condition, with here and there large giant cells, multinucleate, the nuclei occasionally arranged in a semi-circle, staining poorly with hæmatoxylin. The rest of the nuclei appear to be those of feebly staining endothelial cells and of the few lymphocytes with normal staining properties in the periphery of the pale areas, and in a few other spots. The spleen tissue is very hyperæmic, with large blood spaces. A few miliary tubercles in various stages are seen, some with necrosis in the centre. Stained by van Gieson's method (Hansen's modification) Malpighian bodies are not recognisable, and there is little evidence of other normal appearances. Very few arteries can be distinguished. Two cross-sections of a medium-sized vessel show no change. The structure is so obscured that the central artery of the Malpighian bodies can in no case be certainly recognised.

The fibrous tissue is abundant in the altered splenic tissue which separates the areas of poor or absent nuclear staining. In some instances these latter areas are surrounded by a ring of fibrous tissue which penetrates some distance towards the centre, but this penetration and replacement is not so complete or marked as in the glands, where the tubercles are now completely fibrous.

The poorly staining areas with few nuclei correspond to the anæmic areas seen with the naked eye. The structure indicates death of tissue; it is not, however, caseous, nor is it the necrotic tissue with disintegrated nuclei that is commonly seen in the active parts of tuberculous lesions. It recalls most completely the anæmic necrosis seen in infarction, with this difference—that the necrotic tissue, instead of showing faintly the structure of the normal organ, here shows that of a tuberculous lesion, with recognisable endothelial cells and giant-cells. The paucity of vessels may be recalled in this connection.

Summary.—The lesions in all the organs are tuberculous granulomata.

The necrotic areas are neither caseation nor the necrosis with karyorrhexis seen in tuberculous lesions, but a gross anæmic necrosis more often seen in infarction. This anæmic necrosis however, appears to be superimplanted upon a tuberculous lesion. All the lesions, especially those in the liver, show abundant fibrosis, and throughout there is an absence of the lymphocytic reaction seen in active tuberculosis. The presence of giant-cells, together with the increase of fibrous tissue, lend support to the evidence which points to a tendency to heal. In sections of the spleen, liver, lungs and lymphatic glands only a very few tubercle bacilli can be found, *i.e.* some 4 to 12 bacilli per section.

Rabbits.

Four rabbits, 5, 6, 7 and 8, were infected on 12 . i . 23 with living bovine *B. tuberculosis.*

Three of these underwent a course of treatment with "defatted" tubercle-antigen, while the fourth served as a control. Treatment was begun on 15 . ii . 23, *i.e.* 5 weeks after the infection, and up to the present it has extended over three months. The results, which are not yet final, will be given in the form of a short summary of the condition of each animal at the beginning and at the end of the period.

Details of the doses of "defatted" antigen, of the reactions of the animals, their weights, etc., will be found in Tables II, III, IV and IV.

Rabbit 5. (Table II.)

Before treatment.—A prominent nodule was present at the site of infection, and there were some slightly enlarged glands in the left inguinal region.

After treatment.—Both the glands and the nodule had disappeared. The weight had increased considerably.

Rabbit 6. (Table III.)

Before treatment had a well-developed nodule at site of infection, but showed no enlarged lymph-glands.

After treatment the nodule was much reduced in size and felt hard, like fibrous tissue. Its size and characters had not altered appreciably for 3 weeks. The animal's weight underwent considerable increase during treatment.

Rabbit 7. (Table IV.)

Before treatment.—Showed a nodule of moderate size at the site of injection, and also some small inguinal glands.

After treatment.—The nodule had almost completely disappeared, but the glands could still be felt. They were small and hard, and had shown very little change for two weeks past. The animal's weight had increased greatly.

Rabbit 8. (Table V.)

At the beginning of the period.—The infection nodule of this animal had ulcerated through the skin, and it had a large axillary gland.

At the end of the period.—The nodule and the ulcer were stationary. without any tendency to heal. The animal's weight had not increased. At first there was an increase, but then the weight fell again to its original level.

Summary of the Results of Treatment of Animals with "Defatted" *B. tuberculosis* Antigen.

The experiments just described justify, in my opinion, the conclusion that treatment with the "defatted" antigen brings about a definite improvement, both general and local, in animals infected with *B. tuberculosis.* Additional evidence for this view is given by the guinea-pig No. 27 which died, for its organs show what seems indubitably to be a healing of tuberculous lesions.

Of course only time can show whether this beneficial effect is permanent. In order to attain certainty on this question, it will be necessary to watch the animals for a long period without further treatment, and then kill them and make a search for tuberculous lesions in their organs.

EXPERIMENTS WITH B. ANTHRACIS.

In this section will be described complement-fixation, precipitin, agglutination and bactericidal experiments with the sera of—two rabbits, Nos. 12 and 13, immunised with defatted anthrax·antigen; a horse anti-anthrax serum (Mulford); and normal and various immune sera as controls.

I.—Complement-fixation Experiments.

By immunisation with "defatted" anthrax-antigen, complement-fixing substances are produced in definite though not great quantities Table (see XVII).

TABLE XVII.
COMPLEMENT-FIXATION TESTS. ANTHRAX.

DESCRIPTION OF SERUM	QUANTITY OF SERUM IN DROPS *	RELATIVE STRENGTH OF SERUM
NORMAL RABBIT 12. INJECTED WITH DEFATTED ANTHRAX ANTIGEN	2	2·5
NORMAL RABBIT 13 " " " " "	2	2·5
NORMAL RABBIT A	5	1·0
NORMAL RABBIT B	5	1·0
HORSE ANTI-ANTHRAK SERUM (MULFORD)	5	1·0
ANTIDIPHTHERIC HORSE-SERUM 400 UNITS	5	1·0
ANTIDIPHTHERIC HORSE-SERUM 500 UNITS	5	1·0
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	5	1·0

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GAVE COMPLETE COMPLEMENT-FIXATION.

It is of interest to note that the horse anti-anthrax serum, which has high agglutinating and precipitating properties, has no greater power of fixing complement than normal serum. It is also evident that anti-tuberculous and anti-diphtheritic horse sera do not fix the complement, and that, therefore, the effects obtained with the immunised rabbits are specific.

II.—Precipitin Tests. These are shown in Tables XVIII and XIX.

From Table XVIII it is seen that the anthrax precipitinogen, obtained by digesting defatted *B. anthracis* with trypsin, reacts specifically, since it gives a strong precipitation with the horse anti-anthrax serum (Mulford), but no reaction with normal rabbit serum nor with the serum of the tubercle-immune horse.

TABLE XVIII.
PRECIPITATION-EXPERIMENTS. ANTHRAX.

DESCRIPTION OF SERUM	HIGHEST DILUTIONS OF PRECIPITINOGEN SHOWING EQUAL DEGREES OF PRECIPITATION.	RELATIVE ACTIVITY OF SERA
HORSE ANTI-ANTHRAX SERUM (MULFORD)	1/256	256
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/1	1
NORMAL RABBIT 1	1/1	1
NORMAL RABBIT 2	1/1	1

The specificity of the precipitinogen having been demonstrated by means of the serum of an animal immunised with whole *B. anthracis*, it was important to discover whether the injection of "defatted" anthrax antigen into normal rabbits would produce specific precipitins.

TABLE XIX.
PRECIPITATION-EXPERIMENTS. ANTHRAX.

DESCRIPTION OF SERUM.	HIGHEST DILUTIONS OF PRECIPITINOGEN SHOWING EQUAL DEGREES OF PRECIPITATION	RELATIVE ACTIVITY OF SERA
NORMAL RABBIT 12 } INJECTED WITH DEFATTED ANTHRAX ANTIGEN	1/32	32
NORMAL RABBIT 13	1/64	64
NORMAL RABBIT A.	1/1	1
NORMAL RABBIT B.	1/1	1
HORSE ANTI-ANTHRAX SERUM (MULFORD)	1/16	16
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/1	1

The experiments shown in Table XIX clearly prove this point, and show, in addition, that the sera of the rabbits immunised with "defatted" antigen are more active than the immune serum of the horse.

III.—Agglutination Experiments.

In Table XX the results of agglutination experiments, performed with a saline suspension of the "defatted" anthrax bacilli, are given.

The suspension was made up to a suitable opacity with normal saline formolised to 0·1 per cent.

TABLE XX.
AGGLUTINATION EXPERIMENTS. ANTHRAX.

DESCRIPTION OF SERUM	HIGHEST DILUTIONS OF SERA SHOWING EQUAL DEGREES OF AGGLUTINATION.	RELATIVE ACTIVITY OF SERA
NORMAL RABBIT 12 } INJECTED WITH DEFATTED ANTHRAX ANTIGEN	1/2·5	1
NORMAL RABBIT 13	1/50	20
NORMAL RABBIT 1	1/2·5	1
NORMAL RABBIT 2	1/2·5	1
HORSE ANTI-ANTHRAX SERUM (MULFORD)	1/250	100
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/2·5	1

From this table it is seen that the "defatted" antigen is agglutinated specifically by the immune anthrax-serum of the horse (obtained by immunising with the whole bacillus). It is also clear that the defatted antigen, on injection into normal rabbits, is capable of producing specific agglutinins. In the one rabbit, No. 12, no agglutinins had appeared at the time of testing, in spite of 3 or 4 injections with antigen, but the other (No. 13) responded well.

The point raised earlier in the paper, that there is no fixed relationship between the amounts of complement-fixing substances, precipitins and agglutinins contained in an immune serum, is brought out extremely clearly by a comparison of the results given in Tables XVII to XX. Thus it is found that the anti-anthrax horse-serum is the strongest in agglutination, takes a middle place as regards precipitation, and shows no complement-fixation in the quantity of serum used. While rabbit 12 is active in complement-fixation, and shows higher precipitating power than the horse immune serum, it betrays no agglutinating power in the quantities used.

Rabbit 13 gives definite complement-fixation and medium agglutination, while its precipitating power is of a high order.

IV.—Bactericidal Experiments *in Vitro.*

These experiments were carried out with two purposes:

(*a*) Of testing the theory that *B. anthracis* grown at 42° to 43° C. would be more sensitive than an ordinary culture grown at 37° C. to the bacteriolytic or bactericidal effects of normal human serum, and of the serum of a rabbit which had been injected with "defatted" anthrax-antigen.

(*b*) Of ascertaining whether the fresh serum of an animal immunised with defatted antigen contained bactericidal substances for a normal anthrax culture in greater quantities than those found in normal animals.

The details of the experiments are as follows:

Anthrax Bactericidal Experiment I.

Purpose.—To test the relative bactericidal effects of a normal human serum (A.D.G.) on anthrax bacilli grown at 37° C. and on the same culture grown at 42° to 43° C.

The *technique* was in the main that of A. E. Wright, but the results were measured by plating out a measured quantity of each test-mixture on agar-plates, instead of by observing at what dilution growth failed to occur.

The test-mixtures consisted of two volumes of the serum with one volume of a series of dilutions, in broth, of a well-shaken 24 hours' culture (at 37° C.) of an anthrax bacillus (No. I). The dilutions of culture ranged from 1 in 10 to 1 in 1,000,000, with a tenfold increase of dilution at each step.

A control for each mixture was made by substituting broth for the serum.

The mixtures were sucked into capillary pipettes, which, when the tips had been sealed, were incubated for 6 hours at 37° C. At the end of this time 10 cubic millimetres of each mixture were spread over the surface of agar plates, which were incubated for 24 hours.

Result 1/10 to 1/1000, all growths were confluent, but the general appearances suggested a denser growth of the 37° C. bacillus.

At a dilution of 1 in 10,000 the following results were obtained:

37° C. anthrax + serum	About 80 to 100 colonies.
37° C. anthrax + broth	About 500 colonies.
43° C. anthrax + serum	1 colony.
43° C. anthrax + broth	About 100 colonies.

Thus, the reduction in number of colonies under the influence of the serum was 15 to 20 times greater in the 43° growth than in the normal 37° culture.

It is to be noted that the 43° strain, which had been grown at about this temperature for 10 days with daily subcultures in broth, showed a definite decrease of Gram-positiveness.

Anthrax Bactericidal Experiment II.

Purpose.—To test the relative bactericidal effects of the serum of a rabbit immunised with "defatted" anthrax antigen on anthrax bacilli grown (1) at 37° C. and (2) at 42°–43° C.

Technique.—The culture-dilutions were made in sterile dwarf test-tubes with previously calibrated (0·1 c.c.) sterilised capillary pipettes in the following manner: Into a series of dwarf test-tubes 0·9 c.c. of broth was measured and the tubes were autoclaved just before use; 0·1 c.c. of the carefully shaken 18 hours' broth culture was added to the first tube and well mixed with the broth by sucking in and out; then 0·1 c.c. of the mixture was carried over into the second tube, and so on.

Dilutions of 1 in 10 to 1 in 1,000,000 were thus made with each culture (37° and 43° C., grown for 14 hours).

In a fresh series of dry sterile tubes mixtures were made of one volume of each culture dilution with one volume of the serum of a rabbit that had been immunised with "defatted" anthrax antigen. A separate series for the two cultures (37° and 43 C.) was thus set up, and for every tube of serum plus culture a control of broth plus culture, in identical dilution, was prepared.

The control tubes were plated forthwith, without incubation, while the mixtures of serum plus culture were incubated for 6 hours at 37° C. before being plated. The plates were made by spreading 0·02 c.c. of the mixture measured with a calibrated sterilised capillary-pipette on the surface of an agar plate.

The results are shown in Table XXI.

As far as the numbers of colonies are concerned, there seems to be little difference in the susceptibility of the two cultures, though what difference there is indicates greater destruction of the 42° C. bacilli. But a careful microscopical examination, including a count of the individual bacilli, showed that the 42° C. culture was composed of long chains averaging 15 bacilli per chain, while the 37° culture consisted of very short chains of about 3 elements on an average. Since each chain would be expected to start a colony, the number of bacteria destroyed must be reckoned as the number of chains destroyed multiplied by the number of bacilli per chain.

The number of bacilli of the 42° C. culture bacteriolysed must therefore have been some five times greater than the number of 37° bacilli.

TABLE XXI
BACTERICIDAL EXPERIMENT II

ANTHRAX CULTURE (37° AND 42°C.)							
	SERUM OF RABBIT IMMUNIZED WITH DEFATTED ANTHRAX ANTIGEN						
DILUTIONS OF CULTURE	1/1	1/10	1/100	1/1000	1/10000	1/100000	1/MILLION
SERUM AND CULTURES 37°C.	ABOUT 1000	∝	SEVERAL THOUSAND	0	0	0	0
CONTROL OF CULTURES 37°C.	∝	∝	ABOUT 1000	168	20	2	0
SERUM AND CULTURE 42°C.	∝	∝	0	0	0	0	0
CONTROL OF CULTURE 42°C.	SEVERAL THOUSAND	ABOUT 1000	103	17	0	0	0

N.B. 37° AND 42°C. CULTURES WERE MADE FROM THE SAME COLONY OF AN AGAR PLATE · GROWN AT 37°C. CULTURE 37 – GRAM-POSITIVE; CULTURE 42 – GRAM-NEGATIVE – DENSITIES OF CULTURES SEEMED ABOUT EQUAL. THE FIGURES REPRESENT THE NUMBER OF COLONIES WHICH GREW.

Thus the conclusion is justified that growth at 42° C. lowered the resistance of the bacilli to the bactericidal action of this serum.

Anthrax Bactericidal Experiment III.

Purpose.—To compare the bactericidal action, on a normal anthrax culture grown at 37° C., of the serum of a rabbit immunised with defatted anthrax-antigen with that of a normal rabbit serum.

Technique.—*Mutatis mutandis*, the technique was precisely as in the last experiment.

Result.—With culture undiluted, normal serum gave a confluent growth while the immunised serum gave a semi-confluent growth. With culture 1/10, normal serum, about 1000 colonies; immunised serum, about 120 colonies. With culture 1/100, normal serum about 800; immunised serum, 0. With culture 1/1000, normal serum, about 70 colonies; immunised serum, 0.

With higher dilutions of culture no growth occurred in either series.

The serum of the rabbit immunised with "defatted" antigen thus showed itself to be enormously more powerful than the normal serum.

Summary of Conclusions from the Anthrax Bactericidal Experiments.

It has thus been shown in the case of *B. anthracis* that a Gram-positive bacterium, when grown at 42° to 43° C., tends to lose its power of retaining the Gram-stain, and that this change is accompanied by a lowered resistance to the bactericidal action of normal and immune sera.

These experiments afford substantial evidence in support of the explanation offered in the earlier part of this paper (p. 148) of Pasteur's fundamental experiments with "attenuated" anthrax virus.

EXPERIMENTS WITH OTHER BACTERIA.

With respect to bacteria other than *B. tuberculosis* and *B. anthracis*, experiments of a nature similar to those already described are in progress. A short description of the main results obtained up to the present will now be given.

The organisms have mainly been members of the Gram-positive group, *i.e. Staphylococcus aureus*, streptococcus and *B. diphtheriæ*, but a Gram-negative bacillus, *B. typhosus*, has also been subjected to experimental examination.

Staphylococcus aureus.

Defatted antigen has been prepared with this coccus in the manner already indicated. After formol-acetone extraction the coccus is Gram-negative.

It is then, but not before, digestible with trypsin, giving rise to a specific precipitinogen.

Injection of the "defatted" antigen into animals causes the production of complement-fixing substances, precipitins and agglutinins. Growth at 43° to 45° C. can produce a partial or complete loss of Gram-positive staining properties.

Streptococcus.

"Defatted" antigen has similarly been prepared. The "defatted" bacilli are Gram-negative and digestible with trypsin. There are at present no immunity experiments to record.

B. diphtheriæ.

Gram-negative, digestible, "defatted" antigen has been prepared. The digest gives a completely specific precipitin-reaction with antitoxic serum.

Antitoxic sera will also usually agglutinate saline suspensions of the "defatted" antigen. The antigen is specific when used for complement-fixation.

B. typhosus.

In order to show that the "defatting" process does not destroy the specific antigenic substances of a Gram-negative bacterium, *B. typhosus* was subjected to formol-acetone extraction, and rabbits were immunised with several doses of the resulting "defatted" antigen. All the sera thus produced gave positive complement-fixation reactions with the "defatted" antigen. Agglutinins, however, were formed only feebly, or not at all. With the specific precipitinogen, obtained by digesting the "defatted" antigen, the majority of the sera gave a much stronger precipitin-reaction than was shown by a powerful stock typhoid-agglutinating serum. The precipitinogen did not react with normal or immune sera of other types (*e.g. B. paratyphosus A*).

It may be noted that here, again, the absolute independence of agglutination and precipitation is very well demonstrated, which entirely corresponds with S. R. Douglas's findings with digests of acetone-extracted Gram-negative organisms.

TREATMENT OF HUMAN DISEASE.

The fact, now established, that "defatted" antigens of acid-fast, Gram-positive and Gram-negative bacteria could be digested with trypsin gave strong support to the view that they would be similarly susceptible to the disintegrating action of the body-fluids, to which the bacteria, in their normal state, are, in varying degrees, refractory. Additional support to this expectation was given by the fact that the "defatted" antigens caused no local reaction when injected into normal animals.

It seemed, therefore, legitimate to hope that they would prove of value as antigens for the treatment of diseases such as tuberculosis, pyogenic infections, and refractory disorders caused by Gram-negative bacteria such as the gonococcus.

Having shown that small quantities of the antigens were devoid of toxic action, I felt justified in beginning to test their effect, in minute doses, on human patients, and in attempting to discover whether their action would be more favourable than that of the various tuberculins and of vaccines of the usual types.

The proof of such an action could clearly be obtained only by actual treatment of human beings, for animal experiments, however full and convincing, could only prove the case conclusively for the animal species concerned.

Early in April, therefore, acting on my suggestion, Dr. A. C. Inman, of the Brompton Hospital for Diseases of the Chest, inoculated with "defatted" T.B. antigen two patients suffering from active febrile tuberculosis of the lungs and pleuræ. The very small doses employed were determined according to the results of the animal experiments.

The patients, under the care of Dr. Bosanquet, had been under observation for a long period, and were both steadily getting worse. They were selected as being cases only likely to improve if some extra aid could be brought into application. Even in such active cases no local or focal reaction followed the initial inoculation, nor was there any appreciable febrile reaction. Encouraged by the result of the first few inoculations, it was decided to undertake the treatment of a case of acute toxic pulmonary tuberculosis in a young subject, aged 21 years. Again no local, focal or general reaction followed the small initial doses of the antigen; indeed, these resulted in lowering of the temperature and some amelioration of the general condition.

From the nature of the disease under consideration, patient judgment and the lapse of time will be necessary before any opinion can be expressed on the permanent value of the method of treatment. In due time, whatever be the result, a detailed account of all cases treated will be published.

Very shortly after Dr. Inman started treatment of his cases, Drs. P. Fildes and G. T. Western, at the London Hospital, who had followed the results of my researches for some time, and also Capt. S. R. Douglas of the Medical Research Council's Institute at Hampstead, kindly undertook in April of this year to investigate the therapeutic value of the "defatted" antigen of *B. tuberculosis* and certain other bacteria.

Thinking it desirable to avoid any expression of my personal opinion on the matter at the present moment, I have asked Drs. Fildes and Western to send me a short statement of their opinion as to the probable value of this form of treatment, so far as they are able to judge on their present experience. They have been given facilities for testing these antigens at the London

Hospital by the House Committee of that Hospital. Their report is here given verbatim:

"We have under treatment with Dreyer's antigen 60 cases of tuberculosis. Among these are 36 cases of tuberculous adenitis, 11 cases of cutaneous tuberculosis, 4 cases of pulmonary tuberculosis, both active pyrexial and apyrexial, 3 cases of joint and bone tuberculosis, 1 case of peritonitis, 1 case of tuberculosis of the urinary tract, 1 case of iridocyclitis and 3 cases of epididymitis. Most of these cases have been under our personal observation for considerable periods, up to 5 years, and have been selected for treatment as having shown little improvement under treatment with tuberculin B.E. (Koch).

"Improvement has taken place in nearly all cases, and is, in our opinion, of an order which exceeds obviously that obtainable by any other form of treatment which is applicable to these conditions.

"We have not observed any toxic effect following on the inoculations.

"With regard to the effect of these antigens on other bacterial infections, we have treated 6 cases of streptococcal infection such as septicæmia, erysipelas and osteomyelitis, 17 cases of staphylococcal infections, such as sycosis barbæ, furunculosis, etc., and 5 cases of gonorrhœal infection.

"Undoubtedly the course of the disease in many of these cases has been satisfactory as compared with previous experience with vaccine-treated cases. The issue, however, has not always been so straightforward, and therefore an opinion based on three weeks' experience must be more reserved.

"In due course a detailed account of these cases will be published."

In conclusion, I wish to express my warmest thanks to Mr. R. L. Vollum, who has given me assistance of the utmost value and in the most disinterested manner with the preparation of antigens and the performance of the tests.

Also to Dr. A. D. Gardner, who has helped me in a number of ways with experiments, and in the accelerated production of this paper. Further, to Dr. H. K. Ward, who also gave kind help in many experiments; and to Mr. Jesse Wheal, for his careful, skilful and painstaking care of the animals over many months.

In addition, I wish to express my gratitude to Capt. S. R. Douglas, F.R.S., for his helpful interest and invaluable assistance both in animals and materials.

Finally, though not least, to Dr. A. C. Inman, Dr. P. Fildes and Dr. G. T. Western, for their keen interest and their willingness to apply their skill and experience to the testing of my antigens on cases of human disease.

I have also to acknowledge the liberal assistance given to me by the Medical Research Council. Their financial help has greatly facilitated my work.

REFERENCES.

DOUGLAS, S. R.—(1921) "Characters of the Cleavage Products of Bacteria," *Brit. J. Exper. Pathol.*, **2**, 175.

SALIMBENI.—(1912) "Action de certains éthers de la glycérine sur le bacille de la tuberculose," *Compt. Rend. Acad. des Sciences*, **155**, 368.

SANARELLI.—(1920) "La proteide du vibrion cholérique," *Ann. Inst. Pasteur*, **34**, 370.

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OF

EXPERIMENTAL PATHOLOGY

THE ÆTIOLOGY OF RICKETS: AN EXPERIMENTAL INVESTIGATION.—PART II.

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In our previous papers (1918, 1921) we adduced evidence that (1) milk fats and their associated vitamin A have no significant influence in preventing the onset of rickets, or in curing the condition in young dogs; (2) that pups may be reared on separated dried milk and bread without developing the condition; and (3) that pups kept in the open air grow up free of rickets on a diet poorer in milk fats than that of members of the same litter kept in the laboratory which developed the disease. The explanation of these last results is probably to be found in the action of sunlight in preventing and curing rickets and in the influence of temperature and more rapid cooling upon the metabolism.

In their recent paper Powers, Park and Simmonds (1922) state:

> "When the expression 'exposure to direct sunlight' is used, it is inclusive of all that is ordinarily indissolubly associated with sunlight exposure. Sunlight itself, the effect of changes in the atmosphere by solar radiation, temperature, out-of-door air, these and other factors unknown to us, acting singly or in combination, may be responsible for the results usually attributed simply to 'sunlight.'* These experiments bring out, above all else, that exposure to direct sunlight and out-of-door air promotes in a very wonderful way the health, bodily vigour and longevity of animals which otherwise are unable to adapt themselves to markedly adverse environmental conditions.
>
> "Sunlight probably exerts no specific anti-xerophthalmic influence, but acts by raising the level of the cellular activity of the organism to a point where the progress of the disease is held in check or allowed to advance very slowly and with relatively little disturbance."

Our conclusions as regards the want of any action of milk fats is supported by the work of others, although Mellanby's (1919) experiments were accepted by the Vitamin Committee of the Medical Research Council (1919) as demonstrating their anti-rachitic action. One of us (Paton, 1922[2]) has elsewhere analysed his results and has shown that they do not warrant this conclusion.

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* "Experiments are now in progress which seem to indicate that out-of-door air does have a favourable influence upon rats fed on a rickets-producing diet."

The results of Hess, McCann and Pappenheimer (1920–21), and those of Shipley and Park and their colleagues (1921), indicate that butter-fat has no anti-rachitic action, either prophylactic or curative. Sherman and Pappenheimer (1920–21), and Pappenheimer, McCann and Zucker (1922[1]) showed that rats may be reared upon a diet apparently free of any source of vitamin A if sufficient basic sodium phosphate is supplied.

McCollum, Simmonds, Becker and Shipley (1922) found that after oxidative destruction of the vitamin A of cod-liver oil, the residue manifests an anti-rachitic action, but no influence upon xerophthalmia.

Pappenheimer, McCann and Zucker (1922[2]) also have shown that vitamin A in butter or butter-fat neither prevents nor cures rickets.

Zucker, Pappenheimer and Barnett (1922) found that the constituent of cod-liver oil which acts in rickets is in the unsaponifiable fraction of the oil. More recently Zucker (1923) states that it may be extracted by acetone from the calcium soaps and that it is 1000 times as active as the original oil.

That the substance, or substances, in cod-liver oil which, according to McCollum and others, regulates the deposition of bone-salts plays at most a secondary part is shown by the fact that innumerable children and pups grow up free of rickets without the use of cod-liver oil.

The results of these various investigators strongly suggest that any beneficial action which milk may possess on calcification of bone is due rather to the proportion of calcium and phosphorus contained than to the presence of a vitamin.

The fact that sunlight or ultra-violet rays will prevent the onset of rickets upon a diet on which, without them, the disease would appear, is a further indication that the action of these hypothetical vitamins is not essential to the prevention of rickets—that rickets is not a disease due to the deficiency of a vitamin. The hypothesis that the action of sunlight on the skin is to form such specific vitamins seems somewhat far-fetched, and is opposed to the experimental evidence adduced by Hume (1922) and Goldblatt and Soames (1922).

The part played by the adequacy or inadequacy of the supply of calcium and of phosphoric acid in producing ricket-like changes in the bones has recently received considerable attention, chiefly in America (Park, 1923).

The outcome of this American work is to demonstrate firstly the self-evident fact that the limitation of either of these substances leads to the failure of proper ossification, and secondly, to show that the changes at the epiphyses resemble those seen in rickets. How far it is justifiable to identify such a condition with rickets, as seen in children, is, perhaps, questionable. To show that certain morphological changes may be produced by an alteration in the conditions does not necessarily mean that similar changes are always due to these alterations.

True rickets certainly may develop upon an adequate intake of calcium and of phosphorus, and if it is due to a defective supply of these elements to growing bone, it must be ascribed either to decreased absorption from the gut or to the transport of one or other, or both, in unsuitable form in the blood (Paton, 1922[1]). So far there seems no evidence of a defective capacity to fix lime and phosphoric acid in the osteogenic tissue. Grosser's (1920) observations that the retention of calcium may be increased in rachitic children by the subcutaneous administration of calcium glycerophosphates seem to show that the power of fixing the inorganic elements is retained.

The evidence adduced by Telfer (1922–23[2]) seems to indicate that decreased absorption may produce ricket-like changes, but leaves open the question of whether this is the causal factor in the production of rickets.

Whether the action of cod-liver oil and of sunlight in favouring the retention of calcium and phosphorus is upon the absorption by the gut, or upon the metabolic processes by which the elements are fixed, has never been investigated. Our argument (Findlay, Paton and Sharpe, 1921) that the absence of a marked decrease in the calcium of the blood in rickets indicates that failure of absorption of calcium is not the cause of the condition is perhaps unwarranted. The maintenance of a definite concentration of calcium ions in the blood and tissues seems to be essential to normal metabolism, and, even if absorption were interfered with, the stored calcium of bone, or the calcium which ought to go to bone, may be retained in or passed to the blood to maintain that concentration.

That the inorganic P_2O_5 of the plasma is generally reduced in amount has been shown by Howland and Kramer (1921), but to argue from this that the failure of ossification is due to a limitation of necessary P_2O_5 is not justifiable till we have further knowledge of the significance of the normally larger amount of P_2O_5 in organic combination which exists in the blood. The decrease in the inorganic P_2O_5 of the plasma may merely be an accompaniment of some altered metabolism of the phospho-lipoids (Goto, 1922).

It is quite possible that the inorganic P_2O_5 is simply an excretion, as indicated by Meigs, Blatherwick and Cary (1918). This view is supported by the increase of inorganic phosphates which occurs in the plasma in uræmia (de Wesselow, 1923).

McCollum has emphasised the point that it is not so much the absolute amounts of CaO and P_2O_5 in the diet which matters, but rather their proportion, and it is possible that an undue proportion of CaO may lead simply to an increased restriction to the gut of P_2O_5 as phosphate of lime, as has been indicated by Telfer (1922–3[1]), especially on a phosphorus-poor diet.

An increase in the alkalinity of the intestinal content might decrease the absorption of lime and phosphoric acid, and Zucker, Johnston and Barnett (1922) have recently shown that a decrease of the acid base ratio may lead to the development of ricket-like changes in the bones of rats. This is merely an extension of a long series of observations since Seeman's work in 1879 (see Keith and Forbes, 1914).

Telfer's work indicates what Schabad (1910) had previously suggested—that the error of phosphorus metabolism in rickets may be of even greater importance than the disturbance in the metabolism of calcium.

PRESENT INVESTIGATION.

The present paper is concerned with the following questions:

1. The relative importance of restriction of the intake of calcium and of

vitamin A in the production of ricket-like changes in the skeleton; and the retention of calcium and of phosphoric acid on a calcium-rich and calcium-poor diet.

2. The question of how far the action of olive oil in favouring the onset of rickets is due to the absence of a vitamin.

3. The influence of subdued light in favouring the onset of rickets in pups.

4. The possibility of rickets having an infective origin.

(1) *The Relative Importance of Restriction of Calcium and of Vitamin A in the Production of Rickets; and the Retention of Calcium and of Phosphoric Acid on a Calcium-rich and Calcium-poor Diet.*

E. Voit (1880), long before any idea of the possible existence of vitamin A had been suggested, maintained that rickets could be produced in growing dogs by feeding on a diet poor in calcium. One pup was fed upon lean horse flesh with bacon, the other upon the same diet with the addition of bone ash. The former developed rickets, the latter did not, although the supply of vitamin A in bacon fat and in lean meat is now generally accepted to be negligible.

A *résumé* of the more recent work upon the effects of restricted calcium intake is given by Korenchevsky (1922). He states:

> "Therefore I think that a careful examination of the question must lead to the conclusion that in some cases it may be possible by calcium starvation to produce a disease which clinically, chemically, macroscopically and even histologically may simulate rickets. In spite of this similarity, I am convinced that there is an essential difference between the two, and I consider the introduction of the term 'rickets from calcium starvation' (but not pseudo-rachitic osteoporosis) as most desirable. In this group of rickets I propose to include the skeletal changes caused by calcium starvation, or those caused by the introduction of strontium into the organism of animals in a state of calcium starvation. In the latter case there is no osteoporosis, and therefore the term pseudo-osteoporosis is inapplicable."

He concludes that—

> "Other forms of rickets may be produced on a diet containing a normal amount of calcium."

This our previous experiments upon pups has clearly shown to be the case.

The investigations already recorded seem to indicate that the onset of rickets may be retarded by giving an adequate supply of calcium, even when the supply of vitamin A is negligible—that the disease is not necessarily due to deficiency of vitamins either in milk or in cod-liver oil. This conclusion is further supported by the recent work on pigs by Elliot, Crichton and Orr (1922).

A re-investigation of the effects of varied calcium intake with more careful metabolic observations using the more reliable methods of analysis which are now available seemed necessary, since the results of previous investigators are so contradictory (Korenchevsky, 1922).

In our experiments the intake of calcium and of vitamin A was reduced to a minimum by the use of bread and lean meat as a diet. The calcium amounted to only about 0·02 grm. per kilo per day as CaO, while the requirements of a young child, presumably less than those of the more rapidly growing dog, are about 0·15 to 0·2 grm. per kilo. In other pups the calcium supply was made adequate by the addition of calcium lactate to raise the intake of CaO to 0·15 to 0·2 grm. per kilo. In all the experiments the supply of phosphoric acid (P_2O_5) was more than adequate—about 0·12 to 0·17 grm. per kilo.

Experiment X.—This experiment was started when the pups were somewhat too old—8 weeks—and it was of too short duration to yield conclusive results, but it seemed to show that in the absence of any further supply of vitamin than that afforded by lean beef and beef-fat, a pup may develop a normal skeleton for ten weeks on an intake of only 0·02 grm. of CaO per kilo per day.

Three collie pups of one litter which had been on a diet of full-cream milk (dried) and bread were, at 8 weeks old, put in pens on a concrete floor in a room without direct sunlight, and had the following diets, which were increased, to keep the intake per kilo roughly the same, as the pups gained weight.

TABLE I.—*Diet of Pups* (*weights in grms.*).

			Total.	Per kilo.
Pups 1 and 2:				
Full-cream dried milk	33	Fat	7·36	1·96
		CaO	0·566	0·15
Bread	45	Calories	348	92·8
Pup 3:				
Lean beef	54	Fat	14·17	2·71
Bread	100	CaO	0·10	0·02
Beef-fat	9	Calories	574·6	110·0

TABLE II.—*Percentage of CaO and of Fat in Foods used.*

	CaO.	Fat.
Bread	0·08	0·77
Dried milk	1·59	21·0
Beef	0·04	8·16

The growth-curves are given in Chart 1*. No signs of rickets developed. X-ray photographs taken after 7 weeks showed no abnormality in the bones.

The experiments terminated after ten weeks, the dogs being 18 weeks old and having passed the age at which rickets develops. A limitation of lime to 0·02 grm. CaO per kilo of body-weight per day failed to produce the changes described by Voit after eight weeks.

Experiment XV.—The object of the experiment was to test the effects of restricted calcium intake, with and without the addition of vitamin A in butter-fat, in the absence of direct sunlight,

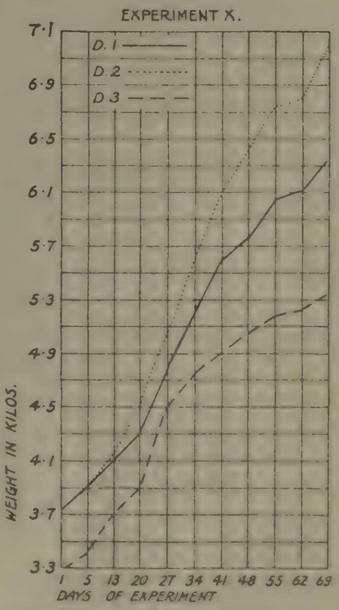

CHART I. WEIGHTS OF PUPS IN EXP. X.

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* In the charts of the growth-curves the day of the experiment is given in the abscissa and the weights in kilos in the ordinates.

and to study the calcium and phosphoric acid balance on a calcium-poor and calcium-rich diet.

Four mongrel pups of the same litter (1 and 3 developed as collies, 2 and 4 as whippet-like dogs) at 7 weeks of age were put in pens, six feet square, in a room without direct sunlight, and had the following diets, which were increased as shown below to maintain the intake per kilo at nearly the same level.

TABLE III.—*Diets (in grms.).*

	Sept. 27th. All diets.	Oct. 19th. All diets.	November 1st.		Nov. 10th. All diets.
			1 and 3.	2 and 4.	
Bread, dry	60	75	85	75	85
Meat	50	60	60	60	70
Butter	3	4	—	4	—

The weight curves are shown in Chart 2. The caloric value of the diet per kilo was between 100 and 130.

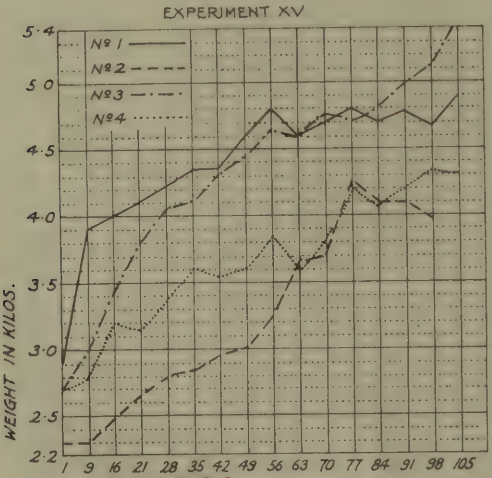

CHART 2. WEIGHTS OF PUPS IN EXP. XV.

After four weeks the diets of pups 2 and 4 contained 2·7 grm. of fat per kilo, and those of pups 1 and 3, 1·4 grm. per kilo. Pups 2 and 3 each had 3·3 grm. calcium lactate (0·6 grm. CaO) per day in addition to the CaO of the food-stuffs (0·068 grm.), *i. e.* they had 0·668 grm. in all per day at the beginning, and 0·96 grm. per day at the end of the experiment. Pups 1 and 4 had 0·068 grm. per day at the beginning and 0·096 grm. per day at the end of the experiment. Pups 2 and 4 had 4 grm. butter in place of 10 grm. of bread.

Slight enlargement of the wrists was observed in No. 1 on November 3rd and the pups were X-rayed, but the photographs showed no marked changes.

On November 22nd the dogs were put in pairs in metabolism cages and the urine and fæces collected for three days.

On November 29th another metabolic experiment extending over three days was made.

The details of these experiments (XVa and XVb) and of a similar metabolic experiment in Experiment XVIII (p. 183) are to be published by Dr. Telfer elsewhere. The main results are shown in Table VII.

On December 3rd X-ray photographs were taken of the fore limbs and showed very slight signs of rickets in pup 1 and pup 4, and no rickets in pup 2 and pup 3, and on December 19th distinct rachitic changes were manifest in pup 1 and pup 4, but no change in pup 2 and pup 3 (Plate I, figs. 3 and 4).

On December 19th pup 2 died under chloroform, and blood was collected from the heart for examination. On December 22nd pups 1, 3 and 4 were killed at the age of 20 weeks. It was noted that the bones of pup 1 were softer than the others.

The examination of the bones gave the following results.

TABLE IV.—*Analysis of Bones (Femora) (weights in grms.).*

Dog.	Weight of dogs.	Weight of femora.	CaO grms. per kilo dog.	Per cent.	P_2O_5 grms. per kilo dog.	Per cent.
Calcium-rich Nos. 2 and 3.	9070	10·4	0·58	24·55	0·46	17·8
Calcium-poor Nos. 1 and 4.	9000	8·9	0·49	22·3	0·40	15·6

It will be seen that the dogs on the calcium-rich diet had appreciably more mineral matter in their skeletons than those on the calcium-poor diet.

TABLE V.—*CaO Content of Muscle (in mg. per 100).*

	CaO.
Pup 2 (calcium-rich)	0·0145
Pup 3 ,,	0·018
Pup 1 (calcium-poor)	0·015
Pup 4 ,,	0·017

There was no appreciable difference in the calcium of the blood of the two pairs of animals.

Experiment XVIII.—This experiment was a repetition of the last, with the intake of calcium increased in two dogs to about eight times the intake of the other two, in the absence of any source of vitamin A other than lean meat and of direct sunlight. The metabolism of calcium and of phosphorus and the gain of each in the skeleton were studied and the distribution of calcium and of phosphoric acid in the blood was investigated.

Four pups, aged 4 weeks, of the same litter were put up on May 2nd, 1922, in pens in a room with a concrete floor into which direct sunlight never fell.

TABLE VI.—*Diet per Dog per day in grms.*

	2nd May to—	15th May to—	22nd May to 11th July.
Bread (dry)	78·5	105	105
Beef (lean)	50	75	77·5

This diet yielded on an average per kilo per day—

CaO	0·03	0·03	0·024	0·016
Fat	1·8	1·8	1·5	1·0
Calories	154	147	117	76

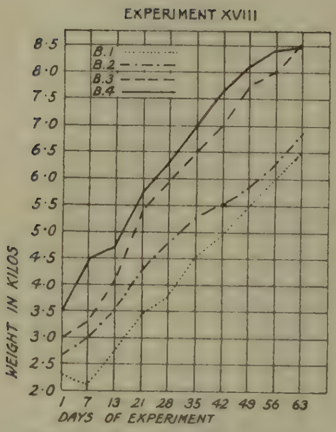

CHART 3. WEIGHTS OF PUPS IN EXP. XVIII

Pups Nos. 1 and 3 had, in addition, 4 grm. calcium lactate=0·73 grm. CaO, giving about 0·28 grm. per kilo, as against 0·03 to 0·016 grm. per kilo in Nos. 2 and 4.

The growth curves are shown in Chart. 3.

Between June 15th and 20th pups 2 and 4 showed weakness of the legs, and on June 21st pup 4 developed tremors resembling tetany, but the electrical reactions were taken and found to be normal.

On June 30th a metabolic experiment of four days' duration was conducted on pups 3 and 4, with the results shown in Table VII. (The details of the experiment are to be published by Telfer.)

Table VII.—*Intake and Retention of Calcium as CaO in the three Metabolic Experiments from Experiments XV and XVIII (calculated in grms. per kilo of body-weight per day).*

	CaO.					
	Calcium-rich (1 and 3).			Calcium-poor (2 and 4).		
	XV(a)	XV(b)	XVIII	XV(a)	XV(b)	XVIII
Intake	0·198	0·16	0·148	0·02	0·02	0·02
Retention (actual)	0·153	0·127	0·14	0·016	0·016	0·02
Retention (per cent. of intake)	77	79	94	80	80	100
	P_2O_5					
Intake		0·12	0·17		0·12	0·16
Retention (actual)		0·086	0·13		0·005	0·056
Retention (per cent. of intake)		72	76		4*	37

On June 22nd X-ray photographs taken showed pups 1 and 3 to be normal, pups 2 and 4 to have signs of rickets (Plate I, figs. 5 and 6).

On July 7th pups 3 and 4 were killed, and on July 11th pups 1 and 2 were killed, age a little over 16 weeks. The bones of pups 2 and 4 were soft.

The longitudinal sections of the ends of the ulna of Nos. 1 and 2 are shown in Plate I, figs. 7 and 8).

Table VIII.—*Calcium and Phosphorus in Blood (in mgs. per* 100 *c.c.).*

	CaO.		P.	
	Whole blood.	Serum.	Inorganic of serum.	Total of whole blood.
Pup 1 (calcium-rich)	10·9	15·3	5·7	42·5
Pup 3 ,,	10·9	14·6	6·0	35·4
Pup 2 (calcium-poor)	11·6	15·2	4·7	27·0
Pup 4 ,,	10·1	14·8	4·8	46·7

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* In dealing with such small quantities, the error in calculating a percentage is, of course, very large.

TABLE IX.—*The CaO Content of the Muscle per cent.*

No. 1	0·014 (calcium-rich)
No. 3	0·014 ,,
No. 2	0·016 (calcium-poor)
No. 4	0·015 ,,

The histological examination of the costo-chondral junction was made by Prof. Korenchevsky, who reports as follows:

No. 1. *Nearly normal.* Zone of prov. calc. normally impregnated with lime. Slight osteoporosis due to the increased number of osteoclasts. Osteoclasts in most places normal. Osteoid is present in physiological amount. Hyperæmia of bone-marrow.

No. 3. *Not very far from normal.* Slightly increased proliferous cartilage and curved line of costo-chondral junction. Very few small sinuses are sometimes present under the line of costo-chondral junction. In a few places amount of osteoid seems to be slightly increased. "Reticulum" bone-marrow in spongiosa.

No. 2. *Rickets from calcium starvation.* Proliferous cartilage greatly increased. Lime deposition in the zone of provisional calcification deficient. Line of costo-chondral junction disorganised, but without deep irregular ingrowth of blood-vessels into cartilage. Large sinuses with thin walls often are present just beneath the line of costo-chondral junction. Trabeculæ of spongiosæ are interlaced and curved, consisting of osteoid tissue with more or less calcified central part. The largest amount of osteoid is equal to or sometimes exceeds the calcified part in the trabeculæ near the line of costo-chrondral junction. In the trabeculæ further from the line of costo-chrondral junction osteoid is present in small amounts. A considerable amount of osteoid is sometimes found under periosteum. Usually there are normal amounts of osteoclasts, but in some places their number is increased. "Reticulum" bone-marrow in spongiosa near the line of costo-chrondral junction. Cartilage canals are also increased in their number.

No. 4. *Rickets.* Picture similar to that of puppy No. 2, but less severe. Proliferous cartilage greatly increased. Sinuses beneath the line of costo-chondral junction. There is no irregular ingrowth of blood-vessels into cartilage. Lime is deposited only in a few places of zone of provisional calcification. Many trabeculæ of primary spongiosa, consisting of osteoid, imperfectly impregnated with lime. In general, osteoid all through, increased, and present in small amount. Osteoporosis.

An examination of the limb bones showed that in the dogs on the calcium-excess diet they were harder than were those in the dogs on the calcium-poor diet. When split they showed well-marked differences in the cortical thicknesses of the shafts. Those from the animals on the calcium-excess (No. 1 and No. 3) were rigid and quite obviously heavier and stronger than the bones of No. 2 and No. 4 (calcium-poor diet), which could easily be bent and twisted. These naked-eye differences of the amount of mineral matter contained in the contrasted specimens were corroborated by the analyses, shown in the following table:

TABLE X.—*Analyses of Bones (Femora) (weights in grms.).*

Number.	Weight of dog.	Femur.				
		Weight per kilo.	CaO.		P_2O_5	
			Per kilo of dog.	Per cent.	Per kilo of dog.	Per cent.
Calcium-rich						
1	6000	1·876	0·425	22·6	0·328	17·5
3	8600	1·811	0·343	18·9	0·259	14·9
Calcium-poor						
2	5900	1·808	0·269	14·9	0·213	11·8
4	8450	1·197	0·158	13·2	0·13	10·8

The ratios $CaO : P_2O_5$ in the bone ash were practically identical in the two series. An excess of calcium in the diet led to an increased fixation of calcium in the skeleton. At the same time a corresponding increased quantity of phosphoric acid was fixed by this calcium, as indicated by the retention results in the metabolic experiments. In all these experiments the amounts of phosphoric acid in the various diets were identical. For conclusions from these experiments see p. 193.

(2) *Olive Oil in the Production of Rickets. Is its Action due to the Absence of a Vitamin?*

Experiment XVII.—The regularity with which all the pups treated by Mellanby with olive oil developed rickets raises the question whether a mere absence of some vitamin is the determining factor or whether olive oil may act in some other way.

To test this, on April 5th a set of four pups at 4 weeks old was used, with four others as checks. We were fortunate in having at the same time 16 pups of two litters of practically the same age, which were used for this and the following investigations.

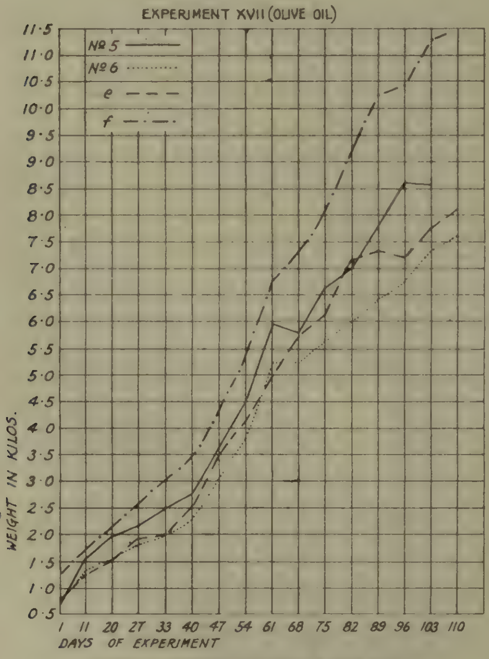


CHART 4. WEIGHTS OF PUPS IN EXP. XVII

Those of the first litter, A, are designated by numbers, those of the second litter, B, by small letters.

The basal diet in all consisted of dried separated milk and dried bread in the proportion of from 22 to 35 grm. of the former and 30 to 40 grm. of the latter, and the calories from 200 to 300 per kilo.

The diets were increased in seven stages as the dogs grew, in order to keep the food supply per kilo as nearly equal as possible. Immediately before an increase was made on account of growth, the energy value of the diet naturally fell below the mean, and immediately after the increase was made the mean of the diet was slightly exceeded.

In four of the pups 10 grm. of the bread were replaced by 5 c.c. of olive oil, keeping the energy value of the diet the same, and after five weeks the oil was increased to 10 c.c. The growth curves are shown in Chart 4.

After 20 days two of the four (Nos. 5 and 6) showed splaying of feet, and X-ray examination indicated ricket-like changes (Plate II, figs. 9 and 11).

A fortnight later No. 6 got 10 c.c. cod-liver oil in place of olive oil and after another fortnight the animal appeared to be better. Nos. 5 and 6 were again X-rayed (Plate II, figs. 10 and 12). A marked improvement was observed in No. 6, but in No. 5 the rachitic process had distinctly advanced at the end of the radius. The pups were killed at the age of 19 weeks, after 14 weeks upon the diet. The general result of the experiment is shown below :

Checks.		Olive oil.
1 normal	3 normal	5 rickets
2 ,,	4 ,,	6 ,,
a ,,	c ,,	e normal
b ,,	d ,,	f ,,

In cutting longitudinally the lower ends of the radius and ulna, the appearance under X-ray was confirmed ; there was marked ingrowth of bone into the epiphyseal cartilage in No. 6, but none in No. 5. The bones of No. 5 were not so hard as those of No. 6 (Plate II, figs. 13 and 15).

TABLE XI.—*Analysis of the Bones.*

Number.	Weight of dog.	Weight of femur.	CaO grams.	CaO per cent. of dog femur.
1	7000	16·41	4·06	24·7%
5	8500	17·15	3·79	22·1%
6	7600	15·35	3·92	25·5%

Prof. Korenchevsky kindly undertook the histological examination of the costo-chondral junctions and I am indebted to him for the following report :

No. 1. Nearly normal.

No. 5. ? Early rickets, increased proliferation of cartilage, osteoid slightly increased, slight osteoporosis, " reticulum " bone marrow.

No. 6. Nearly normal.

TABLE XII.—*Calcium Oxide and Phosphorus in Blood in mg. per 100 c.c.*

	CaO.		P.
Number.	Whole blood.	Serum.	Inorganic of serum.
1. Check	11·74	—	4·9
5. Olive oil (rickets)	11·4	14·8	4·7
6. Olive oil, then cod-liver oil	11·6	14·5	6·2
8. Olive oil (no rickets)	9·4	13·7	5·5

In this experiment the addition of olive oil to a diet of 4 pups upon which 12 pups were reared under exactly the same conditions, free of any signs of rickets, induced the onset of the condition in two out of four animals. The two animals affected were of the same litter.

Manifestly the result cannot be attributed to lack of vitamin in the oil, nor can it be ascribed to a disturbance in the proportions of fats to carbohydrates. On the olive-oil diet these approximate closely to the proportion in which they would occur if whole milk had taken the place of separated milk in the basal diet. Calculated from the basis of 10 grm. of protein, the proportions are:

TABLE XIII.

	Protein (grms.).	Fats (grms.).	Carbohydrates (grms.).
Separated milk diet	10	0·5	28·8
Olive oil diet	10	4·6	24·5
Whole milk diet	10	4·8	31·5

The X-ray pictures and the section of the ulna of No. 5 (Plate II, figs. 9, 10 and 13) further show, in a striking manner, what is well known in children—that the condition may advance in one bone or set of bones and not in others.

A further investigation of the action of various fats upon the absorption and fixation of calcium and phosphorus is urgently required. The older work of Rothberg, Steinitz, Meyer and Orgler from 1903 to 1907 (quoted by Findlay) seems to show that while milk fats have a prejudicial effect on the retention of calcium, cod-liver oil has a beneficial influence.

As already indicated, the more recent work of the American investigators—Hess, McCollum and his co-workers—has fully confirmed these observations, and has shown that the active substance in cod-liver oil, unlike vitamin A, resists prolonged heating and oxidation, so that the oil retains its property of increasing the fixation of calcium after it has lost its protective action against xerophthalmia.

A hint as to the possible way in which olive oil might interfere with the absorption of lime is afforded by the work of Pavloff's school and by the recent work of Lockwood and Chamberlain (1922), which shows a decrease in gastric activity and delay in emptying the stomach when olive oil is taken. This action may increase the alkalinity of the intestinal contents and may possibly interfere with the absorption of lime.

The results of the metabolic experiments (Table VII) showing the very complete absorption of calcium on a calcium-low diet renders an explanation of the mode of action of cod-liver oil and of light extremely difficult.

The absence of any change in the proportion of CaO or of the inorganic phosphates of the blood is clearly shown. The fact that in this and in the succeeding experiment the pups affected were from the same litter emphasises the necessity of using animals of the same litter in such investigations.

(3) *The Influence of Subdued Light.*

Experiment XIX.—We have shown that pups may be reared free of rickets on a diet of bread and separated milk, when kept in a well-ventilated room in the laboratory, exposed to sunlight through an ordinary glass window which filters off the rays which are said to be effective.

It seemed of interest to ascertain if further exclusion from light would produce any effect.

Four pups, aged 4 weeks, were used for this experiment, while the same eight that were used as checks in Experiment XVII were also used in this. All were from the two litters. The pups in one litter are indicated by numbers and those from the other litter by letters.

Checks.		Subdued light.
1	3	7
2	4	8
a	c	g
b	d	9

When put in a subdued light, aged 4 weeks, the pups at once became very lethargic, and, instead of constantly running about in their coop, fighting and barking, as did the pups in sunlight behind glass, they spent most of their time sleeping in a corner. At first they lost appetite, and for 5 weeks did not take all their food.

The growth curves are shown in Chart 5.

After 3 weeks it was noticed that 7 and 8 had enlarged knees, and that g was splay-footed. X-ray photographs at this date were taken (Plate I, fig. 1), and showed—

7 rickets. g normal.
8 ,, 9 ,,

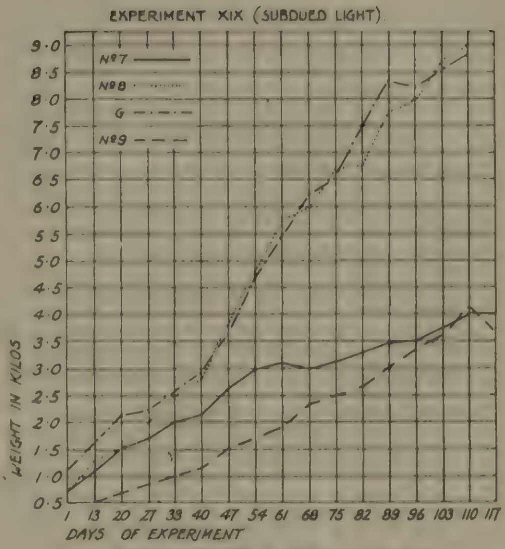


CHART 5. WEIGHTS OF PUPS IN EXP. XIX.

Nos. 7 and 8 were then given 10 c.c. cod-liver oil daily. After 4 weeks their condition was manifestly improved and X-ray examination showed the bones to be normal (Plate I, fig. 2).

The pups were killed on July 17th, at 22 weeks of age. The bones were all hard. Analysis gave the following results:

TABLE XIV.

Number.	Weight of dog.	Weight of femur.	CaO. Grams.	CaO. Per cent.
8	9000	9·36	2·21	23·6
9	4000	7·75	1·94	25·0

TABLE XV.—*The CaO and P of the Blood in Mg. per* 100 *c.c.*

	CaO. Whole blood.	CaO. Serum.	P. Inorganic in serum.	P. Total P in whole blood.
Check	11·7	—	4·9	36·3
Subdued light (rickets)	11·33	14·2	5·5	30·2
Subdued light (normal)	10·1	15·5	5·5	—

One point of no little interest is that while No. 8 showed not only healing of the rachitic process, but also a steady gain in weight, No. 7 showed healing, but little or no gain in weight, while the hair fell out and the coat became thin. Had this dog been alone under treatment, it might have been urged that the improvement was due to the stoppage of growth—an explanation which has been used to explain the improvement in rickets during starvation in rats (McCollum, 1922). But the fact that the same improvement occurred in its brother pup which increased in weight shows that this conclusion is unwarranted.

In this, as in the olive-oil experiment, it was the pups of litter A which developed rickets; that from litter B remained normal, as did its brother and sister in the olive-oil experiment. No more convincing proof could be found of the fact that certain breeds of dogs are more susceptible to rickets than are others, and that it is absolutely necessary in all experiments to compare pups of the same litter.

The experiment seems to indicate that one effect of exclusion from light is to bring about a lethargic condition in pups, and this may play its part in favouring the onset of the disease. From our previous experience on the effects of open-air life in accelerating growth of pups, we are inclined to agree with Powers, Park, and Simmonds (1922) (p. 177).

(4) *The Influence of Infection.*

The association of rickets with unhygienic surroundings and its familial incidence, as well as its mode of onset, progress and recovery, have suggested the possibility of its being primarily of microbial origin. It is not necessary to assume that the infection is a specific one. It might be of the nature of the infection of water which McCarrison has shown to be associated with the development of goitre. It is even possible that a change in the intestinal flora might alter the reaction of the intestinal contents towards the alkaline side and might thus limit the absorption of lime and phosphoric acid.

In our experience, if pups are kept healthy and free from diarrhœa and vermin, they do not develop ricket-like changes in the bones, unless the intake of calcium is markedly cut down. On the other hand, they may develop rickets on an ample intake of calcium and vitamin A in milk, if not kept clean (Renton and Robertson, 1916–17).

Moussu (1903) states that a healthy dog developed rickets when kept in a cage with a rachitic one, and that a second healthy dog, put in the same cage without disinfection, developed the disease. Bull's account (1918) of an outbreak of rickets in fox-hounds strongly suggests an infective element in the onset of the disease.

The evidence of previous investigators has taken the direction of trying to find micro-organisms in the epiphysial region of the bones or of various organs. Such methods do not seem likely to throw much light upon the question and the results are inconclusive. Morpurgo (1900) alone has isolated a diplococcus from the marrow of rats affected with spontaneous rickets, and by injecting it into other rats produced bone changes resembling those of rickets.

In considering how the infective process might be established in the child, it seemed that it might occur either through the blood, by transmission through fleas, lice, or bugs, or by infection through the alimentary tract. The latter view was supported by Marfan (1911). Iovane and Forte (1907) state that they produced rickets in rabbits by injecting extracts of the fæces of rickety children.

An attempt has been made to investigate these possibilities, first, by injecting the blood of rachitic children into pups, and, second, by adding to the food of pups the fæces of children suffering from active rickets.

It is, of course, evident that in such an experiment a positive result would be of great significance, but a negative result would not prove the non-infective nature of rickets.

It may well be that before an infection can be effective, the vitality of the tissues must be lowered by some preceding illness or other condition, and it is also possible that infection from man to dog may not be transmissible, and that canine may not be identical with human rickets.

The following experiments have been performed:

Experiment XIII.—The object of this experiment was to test whether rickets might be produced in pups by the injection of blood from cases of active rickets.

A litter of five pups was put up in two lots at the age of four weeks. In order to obviate the possible criticism, that if rickets developed in any, it was due to a diet with an insufficient supply of vitamin A, the diet was as follows:

TABLE XVI.

	June 15th (grms.).	August 24th (grms.).
Full cream milk (dried) (Cow & Gate)	45	56
Bread (dried)	60	75
Containing :—		
CaO	0·588	0·73
Fat	10·176	12·705
Calories	469	585

The caloric value was, on an average, about 180 to 156, and 135 at the later date, per kilo of body-weight.

From June 22nd pups 1, 2 and 3 got 1 c.c. of blood subcutaneously once a week till July 28th, when No. 2 died, without any signs of rickets, apparently from some interference with deglutition.

By mistake, during August, No. 4 was injected instead of No. 3. Thus all had injection of blood except No. 5.

On September 26th—after twelve weeks—the dogs were all healthy and well-grown and none showed any signs of rickets.

Experiment XII.—This experiment was carried out to find if rickets could be induced in pups by the administration of fæces from cases of active rickets.

A litter of five pups aged four weeks was put up in two lots on May 5th, 1921. The diet selected was—

TABLE XVII.

	May 5th (grms.).	June 29th (grms.).
Full cream milk (dried) (Cow & Gate)	22·5	34
Bread (dried)	30·0	50
Containing :—		
CaO	0·294	0·448
Fat	5·083	7·745
Calories	235	373

The calories per kilo were, on an average, 140 on May 5th and 132 on June 29th.

To the food of Nos. 1, 2 and 3 about 3 grm. of fæces from cases of active rickets were added. Nos. 4 and 5 were used as checks.

X-ray examination on July 12th showed no rachitic change.

They were killed on July 27th.

The bones of Nos. 4 and 5 were softer than those of Nos. 1, 2 and 3, but no signs of rickets were observed.

Dr. Coulthard, of the Pathological Department, kindly made a series of bacteriological examinations of the fæces, and he reports :

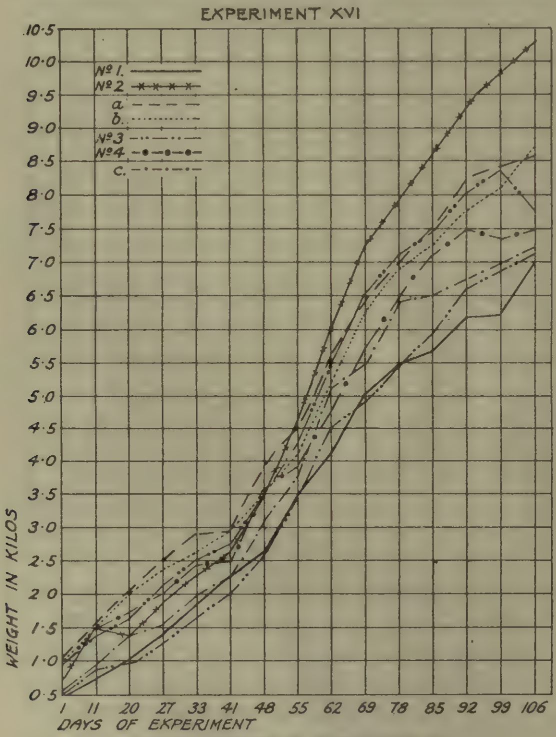


CHART 6. WEIGHTS OF PUPS IN EXP. XVI

"In general, the flora throughout the series showed no great variation, coliform bacilli, streptococci, Gram-positive bacilli of anaërobic types being abundant in all. *B. acidophilus* was present in all, but was most notably abundant in No. 1. Coliform bacilli, as shown by cultures on McConkey's medium, were abundant in all, and when tested for fermentation reactions, etc., were found to be of the common varieties (where there were distinct differences in the appearances of colonies, several were always tested from the same animal). On only one occasion (No. 1) was a non-lactose fermenting bacillus met with; it formed indol, but did not liquefy gelatine, and was somewhat similar to Morgan's bacillus. *Anaërobes.*—There were no striking variations in character or numbers in the series."

Experiment XVI.—This is a repetition of XII, but the vitamin content of the diet was reduced by substituting separated dried milk for full-cream dried milk.

Eight pups from two litters, as described on p. 186, were used, four serving as checks and four getting fæces in their food.

The diet is described on p. 186. All the pups grew well (Chart 6), were lively, and none showed any signs of rickets. The experiment terminated on July 11th, 1922, the pups being then 19 weeks old.

The result of the examination showed that both series were normal.

This series served as checks to the experiments upon olive oil and subdued light (pp. 186–188).

Further experiments upon the possible infective nature of rickets in rats are now in progress.

These experiments do not support the infective theory, but it is very possible that dogs may be resistant to infection from the human subject. The experiments should be repeated with material derived from rachitic dogs.

CONCLUSIONS.

(1) A limitation of the supply of calcium proved to be of more importance in determining the onset of rickets than the limitation of vitamin A, since all other factors except the intake of calcium were the same in the normal and in pups showing ricket-like changes (pp. 180–186).

(2) A very restricted supply of calcium, although leading to changes in the bones, chemically and structurally, resembling those found in rickets, did not materially reduce the calcium of the blood and muscles, or the inorganic phosphorus content of the blood.

(3) The retention of phosphorus appeared to be determined by the retention of the calcium (p. 184).

(4) The percentage of the calcium retained did not vary very markedly with the amount in the food, within the limits investigated of 0·02 to 0·198 grm. CaO per kilo. The very high percentage retention of lime in the calcium-poor diet makes it difficult to explain the favourable action of cod-liver oil upon the retention (p. 184).

(5) A comparison of the condition of pups exposed to sunlight through glass with those from which direct sunlight was excluded showed that the latter condition favoured the onset of rickets, but whether the result was due to absence of the direct action of sunlight or whether it was associated with the lethargy manifested by these pups is not indicated. In this connection, the remarks of Park and his colleagues, given on p. 178, are worthy of attention (p. 188).

(6) The addition of olive oil to a diet which was adequate to prevent rickets actually favoured its onset. This can hardly be explained by deficiency of the oil in some antirachitic substance (p. 186).

(7) Cod-liver oil exercised a marked effect in favouring the cure of rickets in pups (pp. 187, 189).

(8) The addition of fæces from cases of infantile rickets to the diets of pups, whether upon a diet of full cream, milk and bread, or upon a diet of separated milk and bread, was not followed by a development of rickets (p. 191 *et seq.*).

(9) The injection of blood from cases of active infantile rickets did not produce the condition in pups upon a diet of full cream milk and bread (p. 191).

While these experiments give no support to the theory that rickets is an infective disease, they, of course, do not disprove the possibility that a particular condition of the intestinal flora, which might be propagated by fæcal infection, may so modify the absorption of calcium and phosphorus as to lead to rachitic changes.

(10) These experiments further show very clearly that, in testing the influence of any factor in producing rickets, it is necessary (*a*) to use sets of animals and not single animals, and (*b*) to use pups of the same litter.

It has been repeatedly shown that in rats the limitation of the supply of calcium and phosphorus to the bone may produce ricket-like changes, but how the limitation is brought about in infants has never been demonstrated. While we have previously argued against the probability of decreased absorption being the cause of failure in ossification, the work of Telfer and others has shown that decreased absorption cannot be excluded as a factor, and that further study of the intestinal conditions during the development of rickets is required.

We desire to thank Dr. Telfer and Dr. Grace Anderson for the analyses of the calcium and phosphorus of the bones, blood and muscles, which they were good enough to carry out for us.

The expenses of the investigation were defrayed by the Medical Research Council, to which our thanks are due.

REFERENCES.

BULL, L. B.—(1918) *J. Comp. Pathol. and Therap.*, **31**, 193.
ELLIOT, W. E., CHRICHTON, A., AND ORR, J. B.—(1922) *Brit. J. Exper. Pathol.*, **3**, 10.
FINDLAY, L.—(1918) *Med. Res. Committee, Spec. Rep. Ser.*, No. 20, London (H.M. Stationery Office), p. 32.
FINDLAY, L., PATON, D. NOËL, AND SHARPE, J. S.—(1921) *Quart. J. Med.*, **14**, 352.
GOLDBLATT, H., AND SOAMES, K. M.—(1922) *Lancet*, **2**, 1321.
GOTO, KIKO.—(1922) *Tohuko J. Exper. Med.*, **3**, 195.
GROSSER, P.—(1920) *Ztschr. f. Kinderheilk.*, **25**, 141.
HESS, A. F., MCCANN, G. F., AND PAPPENHEIMER, A. M.—(1920–21) *Proc. Soc. Exp. Biol. and Med.*, **18**, 266.
HOWLAND, J., AND KRAMER, B.—(1921) *Amer. J. Dis. Child.*, **22**, 105.
HUME, E. M.—(1922) *Lancet*, **2**, 849.
IOVANE, A., AND FORTE, S.—(1907) *Pediatria*, **15**, 641.
KEITH, M. H., AND FORBES, E. B.—(1914) *Bull. Ohio Agric. Exper. Station*, No. 5.
KORENCHEVSKY, V.—(1922) *Med. Res. Council, Spec. Rep. Ser.*, No. 71. London. (H.M. Stationery Office.)
LOCKWOOD, B C., AND CHAMBERLAIN, H. G.—(1922) *J. Int. Med.*, **31**, 96.
MCCOLLUM, E. V. [ET AL].—(1922) *Johns Hopkins Hosp. Bull.*, **33**, 31.
MCCOLLUM, E. V., SIMMONDS, N., BECKER, J. E., AND SHIPLEY, P. G.—(1922) *J. Biol. Chem.*, **53**, 229.
MARFAN, A. B.—(1911) ‘Le rachitisme et sa pathogenie,’ Paris.
MEDICAL RESEARCH COMMITTEE.—(1919) *Spec. Rep. Ser.*, No. 38. London. (H.M. Stationery Office.)
MEIGS, E. B., BLATHERWICK, N. R., and CARY, C. A.—(1918) *J. Biol. Chem.*, **37**, 1.
MELLANBY, E.—(1919) *Lancet*, **1**, 407.
MORPURGO, B.—(1900) *Beitr. path. Anat. u. z. allg. Pathol.*, **28**, 620.
MOUSSU, G.—(1903) *Bull. Soc. centr. de méd. vét.*, **21**, 303.

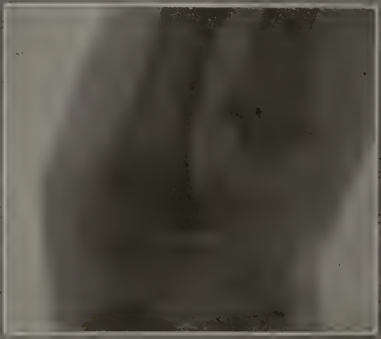
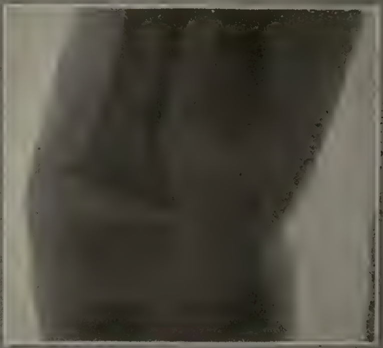


FIG. 9.

FIG. 10.



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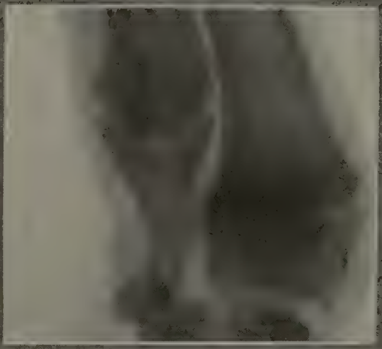

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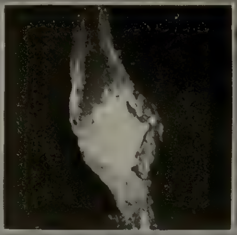

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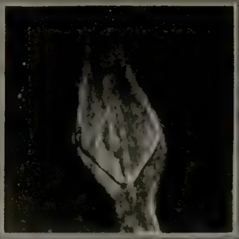

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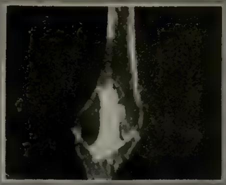
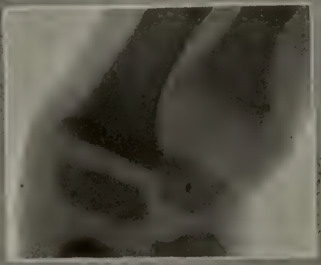


FIG. 15.

Paton and Watson. Plate II.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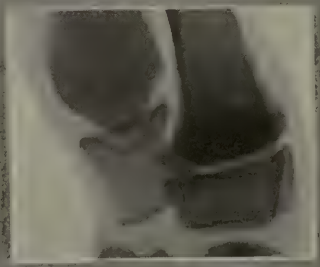
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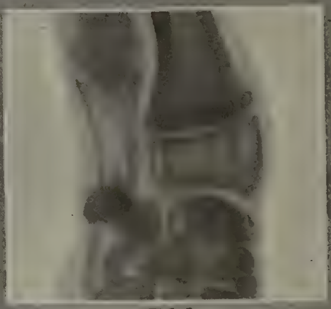
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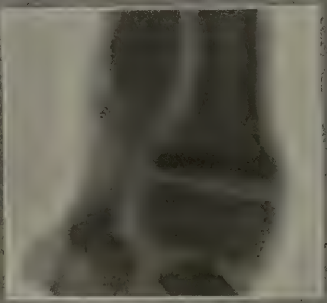
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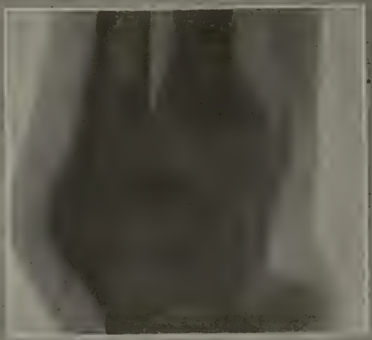
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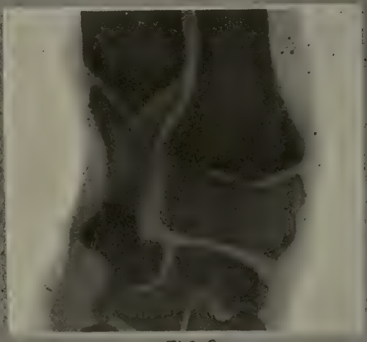
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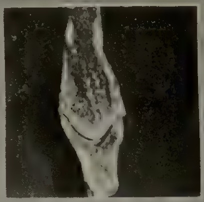
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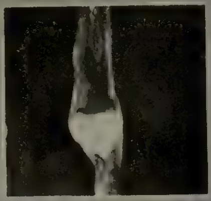
FIG. 8.

Paton and Watson. Plate I.

PAPPENHEIMER, A. M., MCCANN, G. F., AND ZUCKER, I. F.—(1922¹) *J. Exper. Med.*, **35**, 421.—(1922²) *idem.*, **35**, 447.
PARK, E. A.—(1923) *Physiol. Rev.*, **3**, 106.
PATON, D. NOËL.—(1922¹) *Brit. Med. J.*, **1**, 379.—(1922²) *idem.*, **2**, 853.
PATON, D. NOËL, FINDLAY, L., AND WATSON, A.—(1918) *Brit. Med. J.*, **2**, 625.
PATON, D. NOËL, AND WATSON, A.—(1921) *Brit. J. Exper. Pathol.*, **2**, 75.
POWERS, G. F., PARK, E. A., AND SIMMONDS, N.—(1922) *Proc. Soc. Exp. Biol. and Med.*, **20**, 81.
RENTON, J. M., AND ROBERTSON, M. E.—(1916–17) *J. Path. and Bact.*, **21**, 1.
SCHABAD, J. A.—(1910) *Arch. f. Kinderheilk.*, **53**, 380.
SHERMAN, H. C., AND PAPPENHEIMER, A. M.—(1920–21) *Proc. Soc. Exp. Biol. and Med.*, **18**, 193.
SHIPLEY, P. G., AND PARK, E. A. [ET AL].—(1921) *Amer. J. Hyg.*, **1**, 512.
TELFER, S. V.—(1922–23¹) *Quart. J. Med.*, **16**, 45.—(1922–23²) *idem.*, **16**, 63.
VOIT, E.—(1880) *Ztschr. f. Biol.*, **16**, 55.
DE WESSELOW, O. L. V.—(1923) appearing in *Quart. J. Med.*
ZUCKER, T. F.—(1923) *Proc. Soc. Exp. Biol. and Med.*, **20**, 136.
ZUCKER, T. F., JOHNSON, W. C., AND BARNETT, M.—(1922) *idem.*, **20**, 20.
ZUCKER, T. F., PAPPENHEIMER, A. M., AND BARNETT, M.—(1922) *idem.*, **19**, 167.

EXPLANATION OF PLATES.

PLATE I.

FIG. 1.—X-ray photograph of the ends of the radius and ulna from pup 7, Exp. XIX, after 3 weeks in subdued light.

FIG. 2.—The same, after 7 weeks on cod-liver oil, showing cure.

FIG. 3.—X-ray photograph of the ends of the radius and ulna from pup 2, Exp. XV, after 9 weeks on calcium-rich diet.

FIG. 4.—X-ray photograph of the ends of the radius and ulna from pup 4, Exp. XV, after 9 weeks on calcium-poor diet.

FIG. 5.—X-ray photograph of the ends of the radius and ulna from pup 2, Exp. XVIII, after 5 weeks on calcium-poor diet.

FIG. 6.—X-ray photograph of the ends of the radius and ulna from pup 3, Exp. XVIII, after 5 weeks on calcium-rich diet.

FIG. 7.—Photograph of longitudinal section of the end of the ulna of pup 1, Exp. XVIII, on calcium-rich diet.

FIG. 8.—Photograph of longitudinal section of the end of the ulna of pup 2, Exp. XVIII, on calcium-poor diet.

PLATE II.

FIG. 9.—X-ray photographs of the end of the radius and ulna from pup 5, Exp. XVII, after 7 weeks on olive oil.

FIG. 10.—The same after a further 7 weeks on olive oil.

FIG. 11.—X-ray photographs of the end of the radius and ulna from pup 6, Exp. XVII, after 7 weeks on olive oil.

FIG. 12.—The same after 7 weeks on cod-liver oil.

FIG. 13.—Photograph of longitudinal section of the end of the ulna of pup 5, Exp. XVII, on olive oil.

FIG. 14.—The same of pup 8, Exp. XVII, on olive oil, but no rickets.

FIG. 15.—The same of pup 6, Exp. XVII, on olive oil, followed by cod-liver oil.

EXPERIMENTS ON THE RESPIRATION OF MALIGNANT TISSUES.

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THERE is one aspect of the metabolism of tumours which has as yet been investigated very little, viz. the respiration. Although the literature upon the subject of nitrogenous metabolism and proteolytic enzymes is exceedingly extensive and but little perspicuous, the works upon oxidation are few enough to permit a review within a limited space.

In Blumenthal's great compilation, 'Die chemischen Vorgänge bei der Krebskrankheit,' published in 1910, there is not a single line on the subject of respiratory exchange, while Herxheimer and Reinke, in their monograph upon the 'Pathologie des Krebses,' which appeared three years later, could cite only one work on this subject, viz. one by Chisolm (1910), who studied the respiratory exchange of normal and cancerous mice without detecting any difference whatever. Two years before this Cramer (1908) had come to the same result in experimenting with rats.

It is only within the last few years that the respiration of tumour tissues has been studied. Most of this work has been done in the laboratories of the Imperial Cancer Research Fund, London, which has published the four English papers cited below.

Drew (1920) has compared the reducing powers of normal tissues on methylene blue with that of the neoplastic ones, and has come to the conclusion that the oxygen avidity of the tumours is considerably lower than that of normal tissue (kidney, liver, spleen, testis, heart, brain, mamma, connective tissue, "embryo"). His method was very simple: a fine pulp of the tissue was suspended in methylene-blue solution under liquid paraffin at a constant temperature of 37° C.; every half-hour during 5–6 hours the height of the discoloured layer was measured. Drew used solutions of 0·001–0·002 per cent. strength.

This difference between normal and neoplastic tissue was not confirmed by Russell and Gye (1920), who suspended the emulsified tissue (mouse) in rabbit blood saturated with oxygen at 37·5° C., and observed the consumption of oxygen by employing Barcroft's blood-gas apparatus. With every reserve, these investigators draw from their experiments the conclusions that the rate of oxygen consumption rises with increasing speed of growth, and further, that more oxygen is used by tumours which are more highly differentiated histologically.

Russell and Woglom (1920) measured the gaseous exchange of tumours and normal tissue (mouse) by employing Barcroft-Krogh's micro-respirometer. They do not venture upon any conclusions as to the respiration intensity, but they consider that the respiratory quotient of the tumours is proportional to the speed of growth.

Quite contrary to Drew's result, Waterman and Dirken (1921) found a higher oxygen consumption in tumour tissue (*homo*, chicken) than in normal tissue of comparable cellular density (kidney, heart, muscles). They suspended a fine emulsion of the tissue together with red blood-corpuscles in a Ringer solution, which then was saturated with oxygen; at given intervals the oxygen tension was estimated by Barcroft's method.

According to Brahn (1920), cancerous tissue as contrasted with normal tissue possesses no oxidising powers on salicylic aldehyde.

Quite recently Russell (1922) has tested the influence of various carbohydrates on the gaseous exchange of liver, kidney and tumour (mouse) without being able to detect any differences between the various tissues.

An account will be given here of some experiments, thus far preliminary, upon oxidation in human tumour tissue, obtained from the Surgical Clinic of the University, Lund. The oxidation is studied by employing Thunberg's methylene-blue method. As to the arrangement of the experiments I refer to my work, "Über die spontanreduzierende Einwirkung der Muskulatur auf Methylenblau" (Ahlgren, 1921).

The use of the methylene-blue method for studying biological oxidations depends upon the fact that methylene blue is a delicate indicator for the hydrogen which is liberated (activated) from oxidisable substances through the action of the dehydrogenases (Thunberg). Methylene blue as hydrogen acceptor thus plays the same *rôle* as does oxygen in the physiological processes of the tissues. For the theoretical basis of the methylene-blue method, *vide* Wieland (1922) and Thunberg (1917, 1920).

The fitness of the methylene-blue method for the determination of the absolute value of the respiration intensity of a tissue might reasonably be questioned, for its action is not limited to that of a passive hydrogen acceptor, but apparently we have also to do with a catalysis (Meyerhof, 1917, 1918; Thunberg, 1922; Wishart, 1923). But for the solution of the questions treated in this report, as well as for the investigation of many problems concerning the mechanism of respiration, the methylene-blue method has indisputable advantages over gas analysis.

The m-dinitrobenzol method of Lipschitz (1920, 1921), shares with the methylene-blue method the above-mentioned disadvantage (Thunberg, 1922). From the point of view of Wieland's oxidation theory the double nature of m-dinitrobenzol as hydrogen acceptor and oxygen donator should render it a less suitable indicator of oxidation than methylene blue. Moreover, Lipschitz' method cannot be made so sensitive as the methylene blue method and its technique is more complicated. Furthermore, Waterman and Kalff (1923) have recently criticised it severely.

As regards the respiration intensity of tumour tissue, the experiments have proved the extremely great influence of the hydrogen ion concentration and of the presence of an adequate quantity of combustible matter in the tissue.

These factors have been quite overlooked or only insufficiently taken into consideration by previous investigators in this field—a circumstance which lessens the value of their results considerably, as well as explains the contradiction between them. Consideration of these factors is, of course, just as necessary when estimating the respiration intensity of the control tissue.

Very likely the respiratory quotient is also influenced by the factors mentioned.

Another important factor is the degree of mincing of the tissue, concerning which I refer to my report upon oxidation in the muscles, which will shortly be published (Ahlgren, 1923).

It has long keen known that in mincing various animal tissues acid products are formed which are capable of changing the hydrogen ion concentration more or less, a change which decidedly affects the action of the respiratory enzymes. The following experiment illustrates the necessity of regulating the hydrogen ion concentration by means of a sufficiently powerful buffer substance.

Experiment 1, 28 . vii . 1921: *Cancer simplex mammæ* from a 46-year-old woman. Mammary amputation under ether anæsthesia at 9.45 a.m. Soft, medullary, non-necrotic tumour of the size of an orange. A piece of the tumour was freed, as far as possible, from fat and connective tissue, and was minced with a pair of curved scissors.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·25 c.c.	0·25 c.c.
m/10 potassium phosphate	—	0·40 c.c.
Distilled water	0·75 c.c.	0·35 c.c.
Tumour mass	0·30 gr.	0·30 gr.
In thermostat, 37° at	11.40 a.m.	11.46 a.m.
Decoloration time	50 min.	7·5 min.

In two other tubes similar mixtures were placed, but for the methylene-blue solution distilled water was substituted. When brom-thymol blue was added to these tubes, the pH was found in the first one to be below 6·2, in the second one about 7·0.

Probably the accelerating influence of the potassium phosphate on the oxidation may not be exclusively ascribed to its buffer action. The presence of potassium phosphate in a certain concentration seems to be necessary for the normal progress of the cellular respiration (Thunberg, 1909). The normal concentration of the potassium phosphate is, of course, lessened by suspension in methylene-blue solution.

Partly because of its character as a normal cellular constituent and partly because, in contra-distinction to several other buffer substances (*e. g.* citrate, acetate, glycocoll), it cannot be supposed to act as hydrogen donator, potassium phosphate is very suitably employed as buffer substance.

It has long been known that it is necessary to consider the temperature when testing the reaction velocities in physiology. But it is only in recent years that the importance of the hydrogen ion concentration to the enzymatic reactions—for instance to the oxidation in the tissues—has been perceived. Consequently when stating the respiration intensity of a certain tissue one must also tell the temperature and the hydrogen ion concentration at which it was estimated. As regards the temperature, it is natural to use that of the body when working with warm-blooded animals. As to the hydrogen ion concentration, we meet with difficulties as we do not know the pH in the tissues. To use the hydrogen ion concentration at which the action of the enzymes is optimal (for the succino-dehydrogenase about pH = 8·7, according to Ohlsson, 1921) would be unsuitable, as such an alkaline reaction in a living tissue would hardly be conceivable. We find a perfect parallel to this in the temperature, as the most favourable level of temperature for certain dehydrogenases is above that endurable to the organism. For the present it will be most natural to estimate the respiration intensity at a hydrogen ion concentration like that of the blood.

It is astonishing how little the investigators, who have worked upon the respiration of isolated animal tissues, have taken into consideration the error due to the differing percentage of combustible matter. By means of one method or another, one has estimated the gaseous exchange, and a tissue which shows a higher value is simply supposed to have a greater respiration intensity than one that shows a lower value. The fact is overlooked that the lower value may be due to the possibility that the tissue in question has such a great respiration intensity, that the available quantity of combustible matter has been consumed after the separation of the tissue from the system, just as a machine slows down when the supply of fuel is lessened. As is pointed out by Russell, who, in his last work (1922), treats of this source of error, it is well to estimate the gaseous exchange as soon as possible after removing the tissue from the mother organism. But even so, one never knows what influence the source of error has had. Moreover, lack of combustible matter does not necessarily ensue upon the removal of the tissue from the organism. Quite specially in the case of malignant tumours, and perhaps also of certain of the benign ones, one is entitled to look upon such a lack as being possible even *in sitû* in the organism; the spontaneous necrotic decomposition which is here quite frequent might of course be due to deficient supply of food-stuffs. In my work (1921) upon the reducing powers of the muscles on methylene blue I have shown that, in the muscles, the concentration of the substances to which the oxidation enzymes are accommodated very often falls below that at which the enzymes in question act optimally. In my continued investigations I have found that such is also the case in most organs of the body.

It should also be remembered that in the experiment itself a decrease takes place in the concentration of the combustible matter by the suspension of the tumour mass in a bigger or smaller quantity of liquid.

Of course, in order to estimate the oxidising powers of an isolated tissue, one must see to the presence of a sufficient quantity, not only of oxygen (or of some other hydrogen acceptor), but also of the substances that are to be oxidised.

The only way to get a conception of the extent to which the value found for the respiration exchange in a tissue is influenced by a larger or smaller supply of combustible matter, is to estimate the respiration exchange both with and without a surplus of this material added to the medium in which the oxidation takes place. The greater the difference in these two cases, the poorer is the tissue in oxidisable substances.

As to the choice of such available substances for these experiments which could serve as combustible matter in tissues, there are several possibilities.

Glucose and other carbohydrates prove to be unsuitable, as at least most pulped tissues have a very small capacity of consuming them—the amount of insulin present may perhaps here be important. It would be interesting in itself to add a mixture of all the various substances (aliphatic acids, amino acids, aldehydes, etc. (Ahlgren, 1923)), for the catabolism of which the tissues are provided with more or less specific chemical equipment, viz. enzymes of different kinds. Our insufficient knowledge of these substances and of their proper mixing proportions blocks this way. Another way would be to use extracts of fresh organs. Several investigators have applied this method in restituting a metabolism which has ceased because of the consumption of the substrate or because of its elimination by washing. In my work on oxidation in the muscles, mentioned above, I have advantageously used for this purpose an addition of potassium succinate, succinic acid being a substance very easily consumed by most of the organic tissues; it will also fill a very large place in the intermediary metabolism. It has proved suitable also in my experiments with tumours. In some experiments I have also used potassium glycerophosphate (in neutral solution); that, however, unlike the succinate, has the disadvantage of being a strong alkaline buffer substance. Occasional experiments with lactate, acetate, etc., have not succeeded very well.

Here follow the protocols of the experiments in which I have estimated the reducing powers of tumours upon methylene blue, partly without, partly with an addition of potassium succinate or potassium glycero-phosphate. In some of the experiments the reducing powers of muscles from the same patient have been estimated simultaneously—this was done when muscles were extirpated at the same time as the tumour, *e.g.* at mammary amputations. On account of my other duties, two hours at the least, often four or still more have passed between the extirpation of the tumour and the estimation of its reducing powers (transportation, preparations).

EXPERIMENT 2, 12 . i . 1920: *Cancer (gelatinosus) coli sigm.*—Intestinal resection under ether anæsthesia at about 10 a.m. At about 3 p.m. piece of tumour was excised, was washed cautiously in physiological saline, and pulped with a pair of curved scissors.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·5 c.c. (200 γ)	0·5 c.c.
Distilled water	0·5 c.c.	0·5 c.c.
Potassium succinate	—	1 knife's point
Tumour mass	0·2 grm.	0·2 grm.
In thermostat 35° at	3.19 p.m.	3.19 p.m.
at 3.24	—	+*
3.39	(+)	++
3.49	+	+++

EXPERIMENT 3, 5 . vii . 1921: *Sarcoma metastat. reg. lumb.* (round-cell sarcoma) from 59-year-old woman. Extirpation under local anæsthesia at

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* +++ indicates complete decoloration; the other + signs various degrees of decoloration.

9 o'clock a.m. At 11.30 tumour was dissected free, necrotic parts were pared off, remains washed cautiously with physiological saline, slightly dried with filter-paper, pulped with a pair of curved scissors.

Vacuum tube	1	2
Methylene blue 1 : 5000	0·5 c.c. (100 γ)	0·5 c.c.
m/10 potassium succinate solution	—	0·2 c.c.
Distilled water	0·5 c.c.	0·3 c.c.
Tumour mass	0·2 grm.	0·2 grm.
In thermostat 35° at	12.2 p.m.	11.59 a.m.
at 12.30	—	+ +
12.55	—	+ + (+)
1.2	(+)	+ + +
2.50	+ +	—
3.20	+ + +	—
Decoloration time	202 min.	63 min.

Experiment 4, 21 . vii . 1921 : *Cancer* (*simplex*) *mammæ* from 63-year-old woman. Mammary amputation under ether anæsthesia at 10 a.m. Hard tumour; was freed, as far as possible, from fat and connective tissue, was minced and triturated cautiously in a mortar into a fine pulp.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·25 c.c. (100 γ)	0·25 c.c.
m/10 potassium phosphate solution	0·3 c.c.	0·3 c.c.
m/10 potassium succinate solution	—	0·2 c.c.
Distilled water	0·45 c.c.	0·25 c.c.
In thermostat 35° at	12.26 p.m.	12.38 p.m.
Decoloration time	24 min.	14 min.

Experiment 5, 23 . vii . 1921 : *Cancer* (*simplex*) *mammæ* from 65-year-old woman. Mammary amputation under ether anæsthesia at 10.45 a.m. Preparation as in Experiment 4 immediately before the experiment.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·25 c.c. (100 γ)	0·25 c.c.
m/10 potassium phosphate solution	0·30 c.c.	0·30 c.c.
m/50 potassium succinate solution	—	0·05 c.c.
Distilled water	0·45 c.c.	0·40 c.c.
Tumour mass	0·3 grm.	0·3 grm.
In thermostat 35° at	1.15 p.m.	1.19 p.m.
Decoloration time	33 min.	22 min.

Experiment 6, 18 . viii . 1921 : *Cancer* (*simplex*) *mammæ* and of muscular tissue of pectoralis muscle from 36-year-old woman. Mammary amputation under ether anæsthesia at 10 a.m. Rather soft tumour. Preparation as in preceding experiment, only no bruising in mortar. Muscular tissue emulsified with a pair of curved scissors. 37° in thermostat. In each tube 0·4 c.c. of methylene blue 1 : 1000 = 400γ.

Vacuum tube	1	2	3	4	5	6
m/10 potass. phosph. sol.	—	0·4 c.c.	0·4 c.c.	—	0·4 c.c.	0·4 c.c.
m/10 potass. succinate sol.	—	—	0·2 c.c.	—	—	0·2 c.c.
Dist. water	0·6 c.c.	0·2 c.c.	—	0·6 c.c.	0·2 c.c.	—
Tumour mass	0·3 grm.	0·3 grm.	0·3 grm.	—	—	—
Muscle mass	—	—	—	0·3 grm.	0·3 grm.	0·3 grm.
In thermostat at	12.7 p.m.	12.10	12.13	12.26	12.29	12.31
Decoloration time	143 min.	68 min.	38 min.	6 min.	2·5 min.	3 min.

Experiment 7, 16 . xi . 1922: *Cancer* (*medullaris*) *mammæ* and of muscular tissue of pectoralis muscle.—Mammary amputation at about 11 o'clock a.m. Preparation as in preceding experiment. 37° in thermostat. 0·5 c.c. of methylene blue 1 : 2500 in each tube = 200γ.

Vacuum tube	1	2	3	4	5	6	7	8
m/10 potass. phosph. sol.	—	0·2 c.c.	0·2 c.c.	0·2 c.c.	—	0·2 c.c.	0·2 c.c.	0·2 c.c.
m/10 potass. succinate sol	—	—	0·2 c.c.	—	—	—	0·2 c.c.	—
m/10 potass. glycero-phosph. sol.	—	—	—	0·2 c.c.	—	—	—	0·2 c.c.
Distilled water	0·5 c.c.	0·3 c.c.	0·1 c.c.	0·1 c.c.	0·5 c.c.	0·3 c.c.	0·1 c.c.	0·1 c.c.
Tumour mass	0·2 grm.	0·2 grm.	0·2 grm.	0·2 grm.	—	—	—	—
Muscle mass	—	—	—	—	0·2 grm.	0·2 grm.	0·2 grm.	0·2 grm
In thermostat at	2.20 p.m.	2.22	2.25	2.32	2.48	2.54	2.57	3.00
at 5.40	+ +	—	—	—	—	—	—	—
Decoloration time	—	111 min.	53 min.	12 min.	9·5 min.	6 min.	6 min.	6 min.

Experiment 8, 20 . xii . 1922: *Cancer* (*simplex*) *mammæ* and of muscular tissue of pectoralis from 65-year-old man. Mammary amputation about 24 hours before the experiment. Preparation as in preceding experiment. 37°. In each tube 0·5 c.c. of methylene blue 1 : 2500 = 200γ. Buffer substance: 5 c.c. of m/10 K_2HPO_4 solution + 1 c.c. of n/10 caustic potash, 0·3 c.c. of which in each tube.

Vacuum tube	1	2	3	4	5	6
m/10 potass. succinate sol.	—	0·2 c.c.	—	—	0·2 c.c.	—
m/10 potass. glycero-phosph. sol.	—	—	0·2 c.c.	—	—	0·2 c.c.
Distilled water	0·2 c.c.	—	—	0·2 c.c.	—	—
In thermostat	2.31 p.m.	2.33	2.36	2.58	3.01	3.07
at 8 p.m.	+ +	—	—	—	—	—
Decoloration time	>330 min.	96 min.	55·5 min.	42 min.	14 min.	18 min.

In these experiments the importance of the greatest attention to these factors becomes obvious. It is, however, impossible to draw any further conclusions concerning the respiration intensity of malignant tumours. One would almost be inclined to ascribe a lower respiration intensity to the tumour tissue than to the muscles. However, further experiments are needed in this field.

The great increase in methylene-blue reduction caused by the addition of the potassium glycero-phosphate is especially remarkable, although in experiment 7, where the capacity of the phosphate buffer was relatively small, the buffer action of the glycero-phosphate might also have had some influence. As the oxidation of succinic acid and glycero-phosphoric acid is brought about by different enzymes (Ahlgren, 1922), experiments with simultaneous addition of these two substances ought to be made. These acids differ from all other substances hitherto tested that can be oxidised in the tissues, among other things in the extent of their accelerating influence upon the combustion. Also other substances ought to be tested as to their influence upon the tumour tissue in this respect.

Experiment 8 shows that the tumours, after 24 hours of preservation, have lost most of their spontaneous reducing powers upon methylene blue, in which respect they are like liver, kidney, and many other organs (Morgan, Stewart and Hopkins, 1922).

SUMMARY.

When studying tumour respiration one has not hitherto sufficiently considered either the hydrogen ion concentration or the supply of combustible matter in the tissue the respiration of which is to be tested. The importance of these factors is now made evident. The hydrogen ion concentration should be maintained at the normal blood reaction by a sufficiently powerful buffer substance, preferably by potassium phosphate. To estimate the respiration intensity there must be a sufficient quantity present not only of oxygen (or of some other hydrogen acceptor), but also of a combustible matter, this being consumed without replacement when the tissue is removed from the organism. This is suitably done by adding succinic acid or glycero-phosphoric acid, or both of them simultaneously.

REFERENCES.

Ahlgren, G.—(1921) *Skand. Arch. f. Physiol.*, **41**, 1.—(1922) *Compt. rend. Soc. de Biol.*, **87**, 1409.—(1923) *Acta Med. Scand.*, **57**, 508.—(1923) *Skand. Arch. f. Physiol.* (in the press).

Brahn, B.—(1920) *Ztschr. f. Krebsforsch.*, **17**, 417.

Chisolm, R. A.—(1910) *J. Pathol. & Bacteriol.*, **15**, 192.

Cramer, W.—(1908) *Third Sci. Rep. Imp. Cancer Res. Fund*, London, p. 427.

Drew, A. H.—(1920) *Brit. J. Exper. Pathol.*, **1**, 115.

Lipschitz, W.—(1920) *Ztschr. f. physiol. Chem.*, **109**, 189.

Lipschitz, W., and Gottschalk, A.—(1921) *Arch. f. d. ges. Physiol.* (Pflüger), **191**, 1.

Meyerhof, O.—(1917) *Arch. f. d. ges. Physiol.* (Pflüger), **169**, 87.—(1918) *Ibid.*, **170**, 367.

Morgan, E. T., Stewart, C. P., and Hopkins, F. G.—(1922) *Proc. R. Soc.*, B, **94**, 109.

Ohlsson, E.—(1921) *Skand. Arch. f. Physiol.*, **41**, 77.
Russell, B. R. G., and Gye, W. E.—(1920) *Brit. J. Exper. Pathol.*, **1**, 175.
Russell, B. R. G., and Woglom, W. H.—(1920) *Ibid.*, **1**, 244.
Russell, B. R. G.—(1922) *Ibid.*, **3**, 51.
Thunberg, T.—(1909) *Skand. Arch. f. Physiol.*, **22**, 417.—(1917) *Ibid.*, **35**, 163.—(1920) *Ibid.*, **40**, 1.—(1922) *Arch. Néerland. de Physiol.*, **7**, 240.
Waterman, N., and Dirken, M.—(1921) *Ibid.*, **5**, 328.
Waterman, N., and Kalff, J.—(1923) *Biochem. Ztschr.*, **135**, 174.
Wieland, H.—(1922) *Ergebn. d. Physiol.*, **20**, 477.
Wishart, G. M.—(1923) *Biochem. J.*, **17**, 103.

THE RATE AND PROGRESS OF BACTERIOPHAGE ACTION.

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The bacteriophage used in these experiments was isolated from the urine of a woman suffering from bilateral pyelitis due to *B. coli*. It is lytic for the organism grown from the urine of the same patient and for some other strains of *B. coli* as well.

This bacteriophage, when incubated with a broth culture of the autogenous bacillus, produces all the typical appearances which have been described as characteristic of bacteriophage activity. Details of these appearances have been published already (Lepper, 1923).

The active principle has been increased by incubating the bacteriophage in broth with the autogenous culture and subsequent filtration through a Berkefeld filter. After the ninth passage a filtrate was obtained which, in a dilution of 1/2500, was able to reduce to zero an inoculum of 100,000 of the autogenous bacilli per 1 c.c. A heterologous sensitive bacillus required a dilution of 1/500 to destroy completely the same number of organisms.

This sample of bacteriophage was used to test the rate of action of the lytic principle.

Method of Experiment.

The bacteriophage was diluted with peptone-water to a volume of 4·9 c.c.; 0·1 c.c. of an emulsion of bacilli was then added with a dropping pipette, the final volume, 5 c.c., containing the required concentration of bacteriophage.

As soon as the organisms had been added a stop-watch was started, and the mixture was thoroughly shaken for the first half minute. Samples were removed at intervals and drops from a standardised dropping pipette delivered on the surface of a thin layer of set agar in a petri dish. Each drop had a volume of 0·02 c.c.

When the stop-watch showed that the time for plating had arrived, 5 c.c. of melted agar cooled to 42° C. was poured into the dish and thoroughly mixed with the drops of culture.

When this layer had solidified 10 c.c. of melted agar was poured over its surface. The plates were then incubated for 24 hours.

This method of plating was used by Penfold (1914) when studying bacterial lag, and has the great advantage of preventing surface colonies.

The number of drops of the mixture of bacteriophage and bacilli taken for each plating was increased proportionately with the time so as to keep the number of colonies per plate as uniform as possible.

The number of bacilli inoculated was such that all the colonies on each plate could be counted.

In each experiment a control tube containing 4·9 c.c. of peptone-water was inoculated with 0·1 c.c. of the bacillary emulsion and plates were made from it to determine the number of organisms present.

In the charts which illustrate the following experiments the numbers given refer to the colonies which developed per drop (0·02) of the mixtures of bacteriophage and bacilli.

EXPERIMENT I.—*To investigate the rate of action of a dilution of* 1/100 *of the bacteriophage of the heterologous culture.*

The number of organisms inoculated was 12,650 per drop ; the temperature was 21° C.

The results obtained are shown in Chart 1, compiled from figures detailed in Table I.

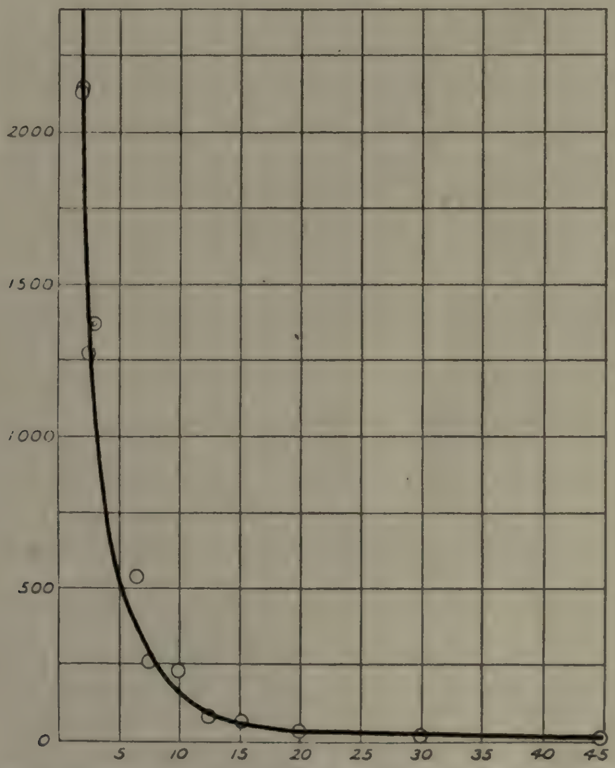

CHART 1.—The rate of action of bacteriophage on heterologous culture. Ordinates = number of bacteria surviving per drop after the action of bacteriophage for minutes (abscissæ).

Table I.

Time of plating.	Number of drops used.	Colonies on plate.	Average per drop.
0 min. 0 sec.	—	—	12,650
1 ,, 40 ,,	1	2,134	2,134
1 ,, 0 ,,	1	2,150	2,150
2 ,, 30 ,,	2	2,544	1,277
3 ,, 0 ,,	3	4,124	1,375
6 ,, 30 ,,	6	3,267	544
7 ,, 30 ,,	8	2,077	262
10 ,, 0 ,,	10	2,283	228
12 ,, 30 ,,	15	1,190	79
15 ,, 15 ,,	20	1,353	67·6
20 ,, 0 ,,	20	622	31
30 ,, 30 ,,	30	460	15·3
45 ,, 40 ,,	40	118	2·9

Discussion of Chart I.—The initial fall in the number of bacilli present is very rapid, the number of colonies per drop at the end of two minutes being only 2150 as compared with 12,650 in the control tube to which no bacteriophage had been added.

After the first five minutes the rate of fall is less rapid and the curve flattens out.

Experiment II.—*To determine the rate of action of various dilutions of the bacteriophage.*

The three mixtures used had the following composition:

A bacteriophage 1/10, bacilli 3130 per drop, temperature 22° C.
B ,, 1/100 ,, 2464 ,, ,, 21·5° C.
C ,, 1/200 ,, 1900 ,, ,, 22·5° C.

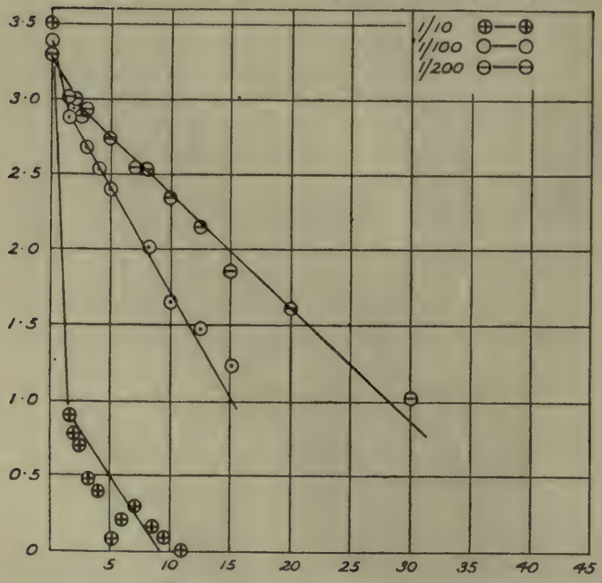

Chart 2.—The rate of action of various dilutions of bacteriophage. Ordinates = logs of number of surviving bacteria.

The results from these three experiments are combined in Chart II. The figures from which the chart is constructed are omitted for brevity.

In this case the logarithms of the numbers are plotted so as to get all the figures into one diagram.

Discussion of Chart II.—The bacteriophage diluted 1/10 reduced the bacilli from 3130 per drop to 8 per drop in $1\frac{1}{2}$ minutes, *i.e.* 99·74 per cent. had been destroyed in that time.

The 1/100 dilution took 15

minutes to reduce the bacilli from 2464 to 16 per drop—a destruction of 99·3 per cent.

The 1/200 dilution took 30 minutes to reduce the bacilli from 1900 to 11 per drop—a destruction of 99·4 per cent.

The initial inoculation in each case was not the same, but the figures show that the 1/200 dilution takes approximately twice as long to reduce the number of bacilli in the same proportion as the 1/100 dilution, and the 1/100 dilution takes about ten times as long as the 1/10 dilution.

The greater the dilution, the less marked is the initial drop in the number of organisms.

In the second stage of the reaction following the initial great fall, the destruction of the bacilli proceeds regularly and the logarithms of the numbers fall on a straight line (5 to 15 minutes).

This period is succeeded by a final phase in which the rate of destruction falls off.

EXPERIMENT III.—*To investigate the effect of temperature on the rate of the reactions.*

In some early experiments the mixtures of bacilli and lysin were kept at 27° C. in a water-bath; when the reaction was found to proceed so rapidly an attempt was made to slow it down by working at a lower temperature.

At 20° C. both the initial fall and the second phase were somewhat less rapid.

An experiment was then carried out in the cold room and the results obtained given in Chart III, where the rate at 0° C. is compared with that found at 27° C. The bacteriophage was diluted 1/100 in each case and the inoculation of organisms approximately the same.

Discussion of Chart III.—The initial drop in the number of organisms is much less at 0° C.

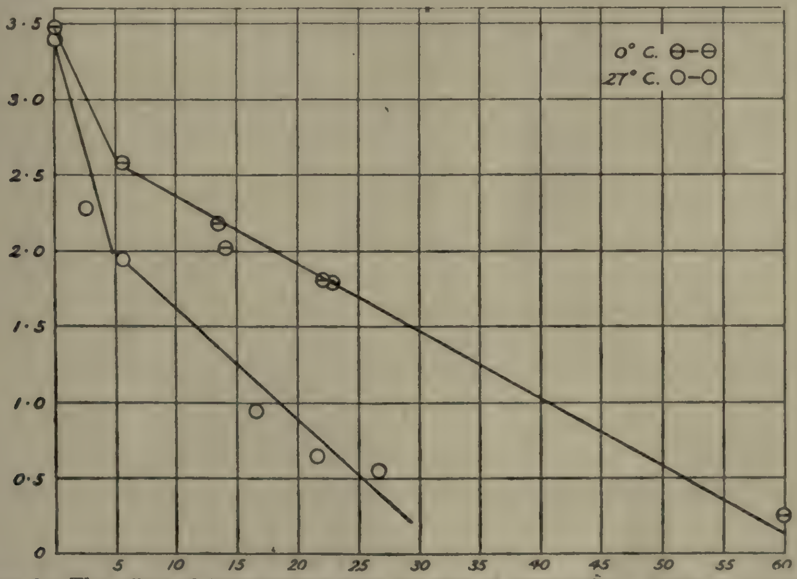

CHART 3.—The effect of temperature upon the rate of destruction. Ordinates = logs of number of surviving bacteria.

The rate is about half as rapid in the second stage.

Even at 0° C. the bacilli are reduced in number from 2935 to 2 per drop in one hour.

Some idea of the order of magnitude of the temperature-coefficient can be obtained by considering the figures of this experiment.

At 27° C. 2465 bacilli were reduced to 192, *i.e.* 7·8 per cent.—in 2·5 minutes. At 0° C., interpolating from the chart, 2935 bacilli were reduced to 229, *i. e.* 7·8 per cent. in 8·5 minutes. The rate being inversely as the time taken, it follows that—

$$\frac{\text{The rate at } 27^\circ\text{ C.}}{\text{The rate at } 0^\circ\text{ C.}} = \frac{8{\cdot}5}{2{\cdot}5} = \frac{3{\cdot}4}{1}.$$

The temperature-coefficient for 10° C. calculated from these figures is 1·57

Considering Experiment II the 1/100 dilution of bacteriophage acting at 21·5° C. took 5·5 minutes to reduce 2462 bacilli to 192 (interpolated), so that—

$$\frac{\text{The rate at } 21{\cdot}5^\circ\text{ C.}}{\text{The rate at } 0^\circ\text{ C.}} = \frac{8{\cdot}5}{5{\cdot}5} = \frac{1{\cdot}55}{1}.$$

The temperature-coefficient for 10° C. from these data is 1·2.

EXPERIMENT IV.—*To compare the action of a 1/100 dilution of the bacteriophage on different numbers of organisms.*

Method used.—Three tubes, each containing 1/100 bacteriophage and the following number of organisms, were prepared:

1. 5,350 per drop.
2. 47,350 ,,
3. 375,000 ,,

These mixtures were kept on the bench at 15° C. and samples were removed at intervals and plated. The results are given in Chart IV.

Discussion of Chart IV.—The lines joining the logarithmic values of the numbers of bacilli surviving in each of the three mixtures at each period of time are parallel.

Hence it follows that the number of bacilli destroyed in a mixture of bacilli and any particular dilution of bacteriophage in a given time depends on the number of bacilli inoculated. At

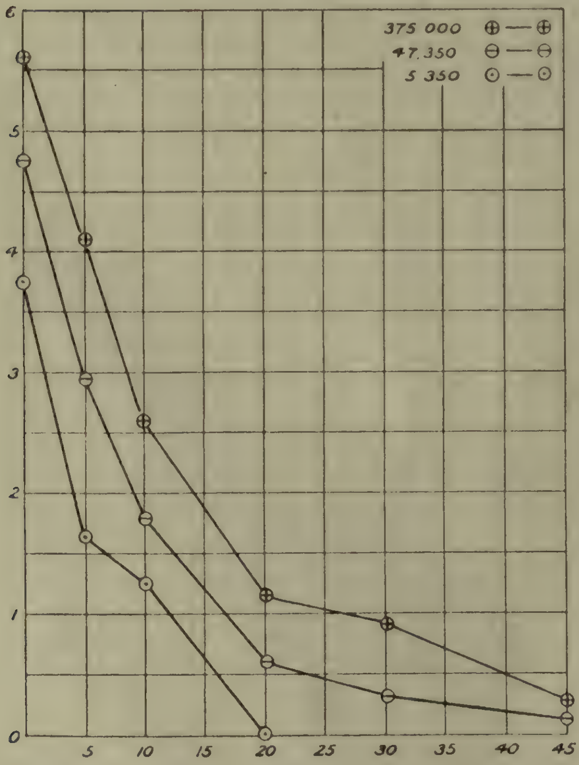

CHART 4.—The rate of action of bacteriophage in the presence of different numbers of organisms. Ordinates = logs of number of surviving bacteria.

the end of five minutes, for example, the number of bacilli killed in the three mixtures was as follows:

1. 5,313, or 99·3% of the inoculum.
2. 46,415, or 98·0% „ „
3. 370,928, or 98·8% „ „

General Discussion of the Rate and Course of the Reaction.

The points representing the numbers of surviving bacilli at different periods in the killing process appear to lie, when plotted, on a fairly smooth curve, but no satisfactory expression has been found for the complete reaction. It is clear from the plotted figures for the logarithms of the surviving bacteria that only the middle or a portion of the middle stage of the process proceeds in the manner of a monomolecular reaction.

The initial stage of lightning killing and the final stages cannot be so expressed, and it is just those stages (and particularly the first) which it is most desirable to be able to analyse. The final stage is complicated by technical difficulties in plating small numbers of residual organisms. When working with such dilutions as 1/100 bacteriophage the carrying over of more than a certain volume of the mixture to the amount of agar used in plating results in a concentration of lysin which can produce a lethal action on the residual bacilli.

With regard to the initial stages of the reaction, the logarithms of surviving bacteria in practically all the experiments cited in this paper and in others not cited lie completely outside any straight line representing a subsequent monomolecular phase. On further analysis, however, it is found that the initial stage finds fairly satisfactory expression by a formula of the bimolecular type in which the velocity of the reaction at any point is proportional to the square of the surviving bacteria; ($\frac{-dy}{dx} = ky^2$, where x = time and y = surviving bacteria).

The constant, k, is calculated from the expression $k = \frac{y_0 - y}{xy}$, where y_0 is the initial seeding.

In the following example (Table II) k has been so calculated and also k' obtained from the monomolecular formula $k' = \frac{1}{x - x_0} \log \frac{y_0}{y}$.

From the fact that a formula of the bimolecular type fits the initial figures fairly closely it would be futile, in the present state of knowledge, to draw any inference as to the nature of the reaction between phage and organisms based on physico-chemical principles. The apparent discontinuity of the reaction, however, suggests that the concentration of the effective substance is altering during the initial stage. With very strong bacteriophage (1/10 dilution), the whole reaction appears to proceed bimolecularly according to the simplified formula $xy = k$, the surviving bacilli at any point being negligible in comparison with the initial inoculum (*i.e.* $y_0 - y$ is practically constant).

TABLE II.—*Bacteriophage diluted* 1/200, *Temperature* 22·5° C.

Time (x).	Bacilli (y).	k (bimolecular constant).		k' (monomolecular constant).	
0′ 0″	1900				
1 30″	1152	0·43			
2′	979	0·46		$x_0 = 2$, $y_0 = 979$	
2′ 30″	762	0·59		·216	
3′	860	0·40		·056	
4′	(402)	(0·90)	Mean k, 0·51	(·193)	
5′	548	0·49		·078	
7′	348	0·63		·09	
8′	339	0·57		·076	
10′	223	·75		·08	
12′ 30″	142	·991		·079	Mean k', 0·078
15′	73	1·76		·086	
20′	41·3	2·38		·077	
30′	10·7	6·3		·071	
62′	1·7	18·05		·046	

On the basis of the disinfectant experiments of Henderson Smith (1921) the discontinuity may be attributed simply to a very skew distribution of resistances in the population presented to the action of the lysin. The question of variation in resistance of the bacilli needs very special consideration in the mode of action of bacteriophage, as of necessity specially sensitive organisms are selected in order that a reaction may take place.

When a specially resistant type of the autogenous bacillus was selected from "nibbled" colonies and treated with the bacteriophage, there was a rapid killing off in the first three minutes of the most sensitive bacilli. After this initial effect there was no further reduction in the number of organisms.

The rapidity of action of bacteriophage recalls the similar phenomenon noted by Rettger and Sperry (1912) in connection with the bactericidal action of egg-white on certain susceptible organisms, and of Chick (1913) on the bactericidal properties of blood-serum, and of Fleming (1922) in connection with the bactericidal action of tears.

In the light of these results, further experiments were undertaken with the object of testing whether bacteriophage became absorbed in the course of the reaction.

EXPERIMENT V.—*To test the activity of a* 1/100 *dilution of bacteriophage immediately after it has acted on varying numbers of bacilli.*

Method A.

Four tubes each containing 4·9 c.c. of 1/100 bacteriophage were prepared. One was kept as a control; the others were inoculated with the following numbers of organisms:

(1) 2485. (2) 29,250. (3) 171,000.

The tubes were kept at 18° C. for 54 minutes. At the end of that time 1 c.c. was removed from each and from the control, and placed in tubes containing 4 c.c. of peptone-water, so that each sample was now diluted 1/500.

At the end of 64 minutes these 1/500 dilutions were plated, the average number of organisms surviving per drop being—

(1) 0·75. (2) 1·5. (3) 5.

At the end of 1 hour 25 minutes each tube received an inoculum of 1282 bacilli per drop. Plates were made at the end of 10, 22, 40 and 94 minutes. The results are shown in Table III.

TABLE III.

Time of plating.	Average number of bacilli per drop in different samples.			
	Control.	(1).	(2).	(3).
0 min.	1282	1282	1282	1282
10 ,,	(1) 484 (3) 372 mean 378	(1) 303 (2) 400 351	(1) 504 (1) 412 458	(1) 137 (1) 438 310
20 ,,	(2) 166 (5) 146 156	(1) 127 (2) 231 179	(1) 186 (2) 128 157	(1) 239 (2) 114 176
40 ,,	(2) 41 (10) 18·6 29·8	(2) 48·5 (5) 26·8 37·6	(2) 39 (5) 33 36	(1) 3 (3) 5·3 4

The number of drops plated are shown in brackets.

Summary of Table III.—Very little difference could be detected between the numbers of bacilli surviving in the different samples at the end of various periods; hence the slowing off of the reaction which takes place during the second phase cannot be explained on the supposition that lysin is adsorbed by the bacilli and put out of action.

Method B.

When the three different numbers of bacilli had been acted on by the bacteriophage for 50 minutes, the three samples, and a control 1/100 dilution of bacteriophage which had not been in contact with bacilli were sterilised by heating at 56° C. for one hour, and the experiment carried on as before. The results are shown in Table IV and Chart V.

TABLE IV.

Time of plating.	Samples of bacteriophage derived from Experiment V_B: numbers of colonies per drop.				
	Control.	1.	2.	3.	Control unheated.
0 min.	3170	3170	3170	3170	3170
17 ,,	631	1322	1686	2470	288
37 ,,	292	398	621	—	29·8
48 ,,	—	—	—	1475	—

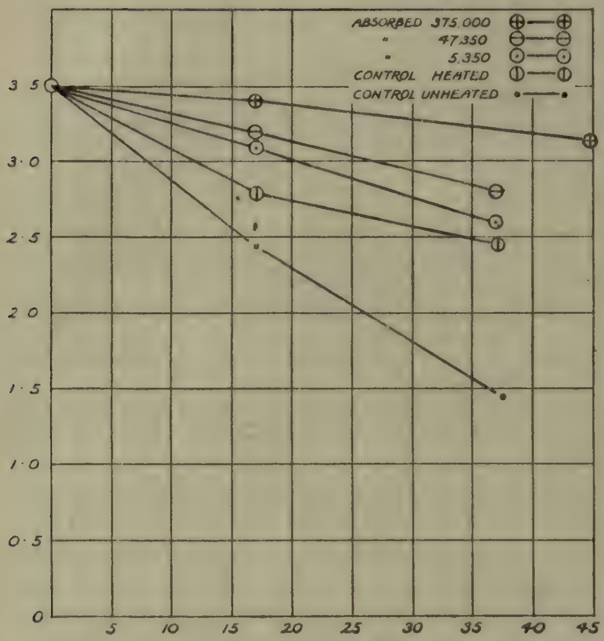

Chart 5.—The rate of action of bacteriophage which has acted on different numbers of organisms and been heated at 56° C. for 1 hour. Controls of heated and unheated bacteriophage which have not acted on organisms. Ordinates = logs of number of surviving bacilli.

Summary of Chart V.—Heating a dilution of 1/500 bacteriophage at 56°C. for one hour reduced its activity as compared with the unheated control.

The heated samples which had been in contact with bacilli showed an even greater loss of activity.

The reduction was greatest with the largest number of bacilli and least with the smallest number of organisms.

The activity of a given dilution of bacteriophage is not reduced by its action on varying numbers of bacilli; if, however, the lysin is heated in the presence of the dead bacilli a reduction in its activity can then be detected.

There is, therefore, no evidence that the slowing off of the rate which occurs during the second phase of the action is due to a disappearance of the lysin. It seems probable that it must be due to the higher resistance of the organisms left in the later stages of the reaction.

GENERAL CONCLUSIONS.

1. The rate of action of bacteriophage on sensitive bacilli is very rapid; 1 in a 100 dilution of the bacteriophage used in these experiments killed off 99 per cent. of the organisms inoculated in five minutes.

2. The rate of destruction of the remaining organisms is much slower; this is not due to a disappearance of the lysin, but is probably to be accounted for by a higher resistance on the part of the remaining bacilli.

3. The rate of the reaction depends on the concentration of the bacteriophage, a 1/200 dilution taking approximately twice as long to produce the same effect as a 1/100 dilution.

4. The number of bacilli destroyed by a given dilution of bacteriophage in a given time varies directly with the number of organisms inoculated.

5. The temperature co-efficient of the reaction is low, the rate at 0° C. being about one-third that at 27° C.

6. Heating bacteriophage at 56° C. for one hour reduces its activity; if bacilli which had been acted on by the lysin during the heating this reduction in activity is more marked.

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REFERENCES.

Chick, H.—(1913) "The Bactericidal Properties of Blood Serum," *J. Hyg.*, **12**, 414.

Fleming, A., and Allison, V. D.—(1922) "Bacteriolytic Elements found in Tissues and Secretions," *Proc. R. Soc.*, B, **93**, 306; **94**, 142.

Henderson-Smith, J.—(1921) "The Killing of Bobrytis Spores by Phenol," *Ann. App. Biol.*, **8**, 27.

Lepper, E. H.—(1923) "On the Mode of Production of Bacteriophage," *Brit. J. Exper. Pathol.*, **4**, 53.

Penfold, W. J.—(1914) "On the Nature of Bacterial Lag," *J. Hyg.*, **14**, 215.

Rettger, F., and Sperry, A.—(1912) "The Antiseptic and Bactericidal Properties of Egg-white," *J. Med. Res.*, **26**, 55.

STUDIES IN EXPERIMENTAL TETANY: I. DISTRIBUTION OF CALCIUM. II. COLLOIDAL AND IONIC CALCIUM.

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THE DISTRIBUTION OF CALCIUM IN THE PLASMA AND CELLS.

Numerous estimations upon the total calcium of blood, of plasma and of serum have been carried out, and while a certain divergence of results still exists, yet the general average arrived at by various workers is practically the same. In their earlier work MacCallum and Voegtlin stated that the percentage of calcium in whole normal blood was about 13·3 mg. while in tetany blood it was about 5·4 mg. Several years later these workers by means of a different analytical method arrived at the conclusion that their former results were too high, and that more correct figures for the percentage of calcium in normal and tetany blood would be 6·1 mg. and 2·7 mg. respectively.

Variations in results are undoubtedly due to difference in the methods used for the estimation of blood calcium. Of the two methods more generally used in recent years, namely, those of Lyman (1917) and Halverson and Bergheim (1917), it seems that the experimental error is much more under control with the former than with the latter. A nephelometric method which depends upon the accurate matching of shades or depth of colour is always open to a personal error, and such method cannot safely be adopted until by practice he who uses it is assured by stringent tests that his readings are within 0·5 to 1 mm. of the correct figure.

In the present investigation Lyman's method has been used and has proved very satisfactory for estimating small amounts of calcium. The nephelometer used was made by taking a Duboscq colorimeter, replacing the cups and plungers with hard rubber cylinders into which short prepared test-tubes fitted accurately.

The test-tubes were supported by means of their flanged edges resting in two lateral slits cut into a hard rubber plate which was screwed on to the colorimeter beneath the prism. The cylinders, supported by movable rings resting on the cup supports, were so adjusted that when the tops were in contact with the hard rubber plate, thereby excluding all light, the indicators on the millimetre scale stood at the zero mark.

The nephelometer attachment was made of wood painted black on the outside and white within. The partition placed between the lamps and the colorimeter contained a plate of frosted glass, and the illumination consisted of one set of two 100-c.p. 110-volt lamps and one set of two 100-c.p. 220-volt lamps, so arranged that any number could be used for illumination.

In determining the distribution of calcium in plasma and cells, it was necessary to obtain accurate values for the proportion of cells to plasma in whole blood. For this purpose it was found that the ordinary hæmatocrit was not satisfactory and the following method was adopted. A piece of pressure glass tubing 10 cm. long having an internal diameter of 1 mm. and a wall thickness of 3 mm. was taken and the ends made smooth with a file. To a 50 c.c. metal centrifuge holder a small right-angled piece of iron was attached so that it formed an inverted L, the arm of the letter being placed over the mouth of the cup and having a tapped hole at the distal end, placed accurately over the centre of the cup. The glass tube filled with blood was placed in a stout rubber band; a small brass cup was placed over the upper end of the tube, the lower end of which was then pressed firmly into the rubber cushion in the bottom of the centrifuge cup by means of a nut which, passing through the tapped hole, fitted into a narrow and comparatively deep hole in the brass cup.

This device was used in duplicate, and after centrifuging the specimen of blood for fifteen minutes very accurate measurements could be made of the proportion of cells to plasma in a length of 10 cm. of blood.

From Table I it will be seen that the average result of the total calcium of the blood of fourteen dogs is 9·12 mg. per cent. In computing this average the figures for Dogs Nos. VIII and IX are not included, as these dogs were suffering severely from mange.

The average figure for the amount of calcium in plasma from 100 c.c. of blood is 8·11 mg., while for the corpuscles from the same amount of blood it is 1·01 mg.—that is to say, in a hundred parts by volume of blood we find a distribution of calcium between the plasma and the cells in a proportion aproximately of 8 to 1.

In a series of some twelve rabbits in which similar estimations were made it was found that the figures were slightly higher, being for whole blood 10·0 mg. per cent., for plasma from 100 c.c. of blood 8·6 mg., and for the cells 1·4 mg.

With the parathyroidectomised dogs estimations were made usually on the second day after operation. If the animals survived for two or three days longer, then a second determination was made. On several of these animals a partial parathyroidectomy was performed, but no conclusive results were obtained immediately following such an operation, and the results quoted are those in totally thyro-parathyroidectomised animals. The pH was estimated on the day following operation on blood drawn from the left ventricle, the method used being that of Levy, Rowntree and Marriott (1915).

TABLE I

DISTRIBUTION OF CALCIUM IN THE CELLS AND PLASMA OF NORMAL DOGS.

DOG	pH	TOTAL Ca mgs %	HÆMATOCRIT READING mm.	PLASMA Ca PER 100 cc. BLOOD	CELL Ca PER 100 cc BLOOD	CELLULAR Ca % OF TOTAL
I	7·30	9·60	3·7	8·36	1·24	12·19
II	7·30	8·10	3·6	7·20	·90	11·1
III	7·30	8·00	8·4	7·36	·74	9·3
IV	7·30	8·00	2·8	7·28	·72	9·0
V	7·35	7·75	3·4	6·79	·96	12·2
VI	7·35	8·10	3·1	7·24	·86	11·6
VII	7·35	10·00	3·1	8·83	1·17	11·7
VIII	7·30	8·20	3·2	7·80	·40	4·9
IX	7·20	7·50	3·2	7·20	·30	4·0
X	7·30	7·85	3·5	6·95	·90	10·2
XI	7·25	9·12	3·5	8·25	1·87	9·6
XII	7·25	9·60	3·0	8·40	1·20	12·5
XIII	7·25	10·80	2·7	9·56	1·24	11·5
XIV	7·30	11·00	3·4	9·90	1·10	10·0
XV	7·30	9·70	2·8	8·85	·85	8·8
XVI	7·30	10·00	2·5	8·70	1·30	13·0

TABLE II

DISTRIBUTION OF CALCIUM IN THE CELLS AND PLASMA OF TETANY DOGS.

DOG	pH	TOTAL Ca mgs. %	HÆMATOCRIT READING mm.	PLASMA Ca PER 100 cc BLOOD	CELL Ca PER 100 cc BLOOD	CELLULAR Ca % OF TOTAL
I	7·40	5·82	3·0	5·32	·50	8·5
II	7·50	5·37	1·9	4·86	·51	9·5
III	7·45	5·62	2·8	5·19	·43	7·6
IV	7·53	5·75	3·2	5·16	·51	10·26
V	7·40	6·50	8·5	6·04	·46	7·1
VI	7·50	6·25	3·3	5·83	·42	6·7
VII	7·40	6·50	3·6	6·10	·42	6·2
X	7·45	4·93	3·5	4·45	·48	9·7
XI	7·45	4·75	2·7	4·41	·34	7·1

NORMAL DOGS AVERAGE pH = 7·293
TETANY DOGS AVERAGE pH = 7·455
pH DIFFERENCE 0·162

TABLE III

COMPARISON OF AVERAGE FIGURES FOR CALCIUM DISTRIBUTION IN NORMAL DOGS AND TETANY DOGS.

Ca % IN mgs. WHOLE BLOOD	PLASMA Ca mgs. PER 100 cc OF BLOOD	PLASMA Ca % OF TOTAL CALCIUM	CELL Ca PER 100 cc BLOOD	CELL Ca % OF TOTAL Ca	REMARKS
9·12	8·11	88·9	1·01	11·1	NORMAL DOGS
5·72	5·26	91·9	·46	8·1	TETANY DOGS
37·4	35·2		54·4		PERCENTAGE FALL IN Ca

Taking seven completely thyro-parathyroidectomised dogs, the average figures, quoted in Table II, show a whole blood calcium content amounting to 5·72 mg. per cent., with a plasma and cell content per 100 c.c. of whole blood of 5·26 mg. and 0·46 mg. respectively. It is thus seen that in experimental tetany the ratio of plasma calcium to cell calcium is approximately 11·5 to 1·0.

The alteration in the calcium distribution may be more clearly seen from Table III, in which the relative percentage of calcium in the plasma and cells to the total blood calcium is given, and in which the comparative alterations are indicated as percentages of the normal contents. It is here shown that in the condition of tetany there is a considerable loss of cell calcium amounting to 54·4 per cent. as compared with a plasma loss of 35·2 per cent.

DIFFUSIBLE AND COLLOIDAL CALCIUM IN NORMAL AND THYRO-PARATHYROIDECTOMISED DOGS.

In 1916 a method for the estimation of diffusible calcium was introduced by von Mysenbug and others (1916). This method is one of compensation dialysis, in which the diffusible salt in the dialyser is balanced by the addition of an equal amount of the salt to the dialysing fluid.

The separation by filtration of the diffusible salts of blood-serum can be effected by subjecting the filter to a pressure of 150 mm. of Hg. This method was employed by Cushny (1920) ; but to determine accurately the amounts of a salt in ionic and colloidal form it is essential not to alter the balance between them by removal from the solvent of the diffusible salt. According to the law of mass action the removal of the ionic calcium would result in a further breakdown of calcium in combination in order to restore the normal balance. For this one reason, therefore, apart from others, the compensation method of dialysis is the better and has been made use of in this work.

While a considerable drop in the total calcium of the blood and of its cellular elements has been noted in parathyroid tetany, the question as to whether or not this was associated with a loss in free or combined calcium has not been definitely settled. That alterations may occur in the diffusible calcium in rickets and in tetany was suggested by von Mysenbug and his co-workers, but in a series of four experiments they found no distinct variation from the normal.

METHODS.

Preparation of parlodion sacs.—The shreds of parlodion are dried in the air and dissolved in ether and 95 per cent. alcohol in the required proportions. After solution of the parlodion shreds is complete, the bottle, firmly stoppered, is allowed to stand for two weeks.

Two small test-tubes, 15 mm. internal diameter and 60 mm. long, are filled with the mixture, centrifuged to bring all air-bubbles to the surface, the hardened surface then peeled off and the contents emptied into the stock bottle, the tube being rotated meanwhile in order to secure uniform thickness of the wall of the sac. The tube is clamped in the inverted position for 15 minutes, then placed in cold distilled water for half-an-hour. The upper rim is loosened by means of a knife, and a thin glass rod with a bulbous tip gently pushed between the sac-wall and the glass to the bottom of the test-tube, which is kept half filled with water. The sac and the glass rod are withdrawn together and the sac immersed in cold distilled water.

Compensation dialysis.—In view of the fact that in paratyphoid tetany variations in diffusible calcium may be great or small, it was necessary to make up a series of dialysing calcium solutions, in order to balance as nearly as possible the amount of free calcium found in the serum. For this purpose ten dialysing solutions were made up, giving a percentage of normal serum calcium varying from 40 to 80. These solutions were made from a stock solution of calcium chloride of about 1 per cent. strength, the amount of calcium in the stock solution having been determined by the nephelometric method. The following is the series of solutions employed:

COMPENSATION DIALYZING SOLUTIONS.

SOLUTION	AMOUNTS OF Ca IN mgs. IN 2·5 cc.		AMOUNTS OF Ca IN mgs. %		PER CENT. OF NORMAL Ca
	ESTIMATED	CALCULATED	ESTIMATED	CALCULATED	
A	·206	·193	8·25	7·71	39·28
B	·213	·210	8·54	8·40	40·65
C	·243	·241	9·75	9·65	46·42
D	·257	·262	10·30	10·30	49·10
E	·290	·289	11·62	11·58	55·33
F	·312	·315	12·50	12·60	59·52
G	·343	·338	13·75	13·50	65·47
H	·367	·363	14·68	14·70	69·90
I	·390	·355	15·62	14·19	74·38
J	·406	·420	16·25	16·70	77·38

To 2·5 c.c. of the solution employed 2·5 c.c. of a double strength dialysing fluid were added, the composition of which is as follows:

DIALYZING FLUID: (GREENWALD)

SALT	PER CENT AMOUNTS SINGLE STRENGTH	MOLAR STRENGTH	AMOUNTS IN 2000 cc. DOUBLE STRENGTH
$NaHCO_3$	·2520 gm.	·003	10·08 gm.
KH_2PO_4	·0088 gm.	·000065	12·9 cc M/5
$MgCl_2$	·01059 gm.	·00011	0·2118 gm.
KCl	·02960 gm.	·000397	1·184 gm.
NaCl	·6250 gm.	·01069	25·0 gm.

Since the balance between diffusible and colloidal calcium is in some measure dependent upon the H-ion concentration of the serum, it is essential to bring the CO_2 tension of the serum into equilibrium with an atmosphere of CO_2 of a tension approximating that of alveolar air. To do this a large spirometer was filled with CO_2 and air; these were thoroughly mixed, and the percentage of CO_2 estimated daily by the Henderson and Morris (1917) method.

The gas was passed through a bottle containing moist glass beads, then through two separatory funnels placed in series, the first containing about 20 c.c. of serum, the second about 10 c.c. of the double strength dialysing fluid. This equilibration was carried out at room temperature, which during the course of these experiments varied from 30° to 33° C.

The dialysis.—The sacs to be employed were thoroughly washed in distilled water, shaken free of water and allowed to drain, but not allowed to dry in the air. The test-tubes used for dialysis were ¾ in. wide by 4 in. long, and into these were placed 2·5 c.c. of the double strength dialysing fluid, and CO_2 passed into the test-tubes for a few minutes.

The sacs, supported on small hard rubber cylinders, were filled with 5 c.c. of serum, CO_2 was passed into the sacs, a small cork firmly inserted into the top of each cylinder, and the whole supported in the test-tube. Dialysis was carried out for 24 hours at a temperature of 12° to 15° C.

DISCUSSION OF RESULTS.

In the following series of tables, Nos. IV to VII, the results obtained in normal, partially, and totally thyro-parathyroidectomised dogs are given.

TABLE IV.

TOTAL SERUM CALCIUM IN NORMAL DOGS, IN PARTIALLY, AND IN COMPLETELY PARATHYROIDECTOMIZED DOGS.

NORMAL DOGS	DOGS PARTIALLY PARATHYROIDECTOMIZED.	DOGS COMPLETELY THYRO-PARATHYROIDECTOMIZED.
11·24	6·25	5·00
10·50	6·13	5·10
11·20	8·20	5·00
11·30	AVERAGE 7·04 mgs.	4·70
12·70		5·20
12·60		4·60
9·00		5·40
AVERAGE 11·30 mgs. (100%)	(DROP = 37·72%)	AVERAGE 4·95 mgs. (DROP = 56·20%)

Table IV shows the variations in percentage of the calcium content of serum under the three specified conditions. From a normal average of 11·30 mg. per cent., the calcium fell in partially parathyroidectomised animals to an average of 7·04 mg. per cent., to be followed by a further fall upon complete parathyroidectomy to an average figure of 4·95 mg. per cent. It is thus seen that the loss of calcium from the serum amounts to 56·20 per cent. of the whole.

With regard to diffusible calcium the figures quoted in Table V, comprising results obtained from a series of seven normal dogs, are similar to those of Cushny and von Mysenbug, namely that in normal animals the diffusible calcium amounts to between 60 and 70 per cent. of the whole calcium content of the blood, taking 10·5 mg. as the percentage of calcium in normal blood-serum.

In comparing the results recorded in Tables VI and VII from partially and completely thyro-parathyroidectomised dogs respectively, it is seen that there is no essential difference in the amounts of calcium recorded.

It is somewhat difficult to say to what extent a single parathyroid gland may or may not be functioning. The upper left parathyroid was left with a small piece of thyroid tissue. In all of these cases the gland retained was distinctly identified, but we must admit that this method of leaving a gland *in sitû*, while all other similar tissue is removed, is not, *par excellence*, the method that should be followed in the investigation of the internal secretion of any gland. Just as in partial depancreatisation, so in partial parathyroidectomy, the gland or piece of gland should be transplanted and the activity of the graft definitely proved before it is totally removed. In the former method one cannot, with assurance, state whether or not there has been any alteration in the normal blood- and lymph-supply due to operative interference. In all of these operative animals subcutaneous suture was employed, no dressings were applied, and without exception excellent healing of the neck wounds was obtained.

From the average percentage of diffusible calcium recorded for these tetany animals, namely, 93·70, the individual percentage being computed from the total amount of calcium in each sample of serum, it is evident that the loss of colloidal calcium is indeed a marked one.

It is true that the compensation in dialysing was not complete; but in view of the high percentage recorded, and also in view of the result obtained with the final serum from Dog VIII, in which no compensation was attempted, one is forced to the conclusion that in severe tetany the major part, if not all, of the colloidal calcium is freed from its protein combination. It would also seem from certain of the results that it would be possible to determine the rate of the breakdown of this calcium proteinate linkage.

RESULTS OF COMPENSATION DIALYSIS OF SERUM CALCIUM.

TABLE V. NORMAL DOGS.

DOG I. TOTAL Ca = 10·5 mgs.%: Ca IN 5 cc. SERUM = 0·525 mgs.: CO_2 TENSION = 49·64 mm. Hg.

Ca IN mgs. IN 5 cc. SERUM		Ca IN mgs. IN 5 cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·525	·584	·343	·317	55·6	G
·525	·534	·343	·316	55·4	G
·525	·493	·312	·332	67·1	F
TOTAL Ca = 11·24 mgs.%: Ca IN 5 cc. SERUM = 0·562 mgs.: CO_2 TENSION = 50·16 mm. Hg.					
·562	·503	·312	·337	64·4	F
·562	516	·343	·413	61·4	G
·562	·462	·367	·383	70·6	H

DOG II. TOTAL Ca = 10·5 mgs.%: Ca IN 5 cc. SERUM = 0·525 mgs.: CO_2 TENSION = 52·92 mm. Hg.

·525	·530	·343	·344	65·5	G
·525	·515	·367	·354	64·9	H

DOG III. TOTAL Ca = 11·7 mgs.%: Ca IN 5 cc. SERUM = 0·585 mgs.: CO_2 TENSION = 50·92 mm. Hg.

·585	·505	·312	·342	63·5	F
·585	·558	·343	·362	65·1	G
·585	·550	·367	·382	67·9	H
·585	·557	·367	·377	66·1	H

DOG IV. TOTAL Ca = 11·30 mgs.%: Ca IN 5 cc. SERUM = 0·565 mgs.: CO_2 TENSION = 44·14 mm. Hg.

·565	·480	·312	·343	66·1	F
·565	·491	·343	·323	59·1	G
·565	·511	·367	·362	63·1	H
·565	·537	·367	·372	66·7	H

DOG V. TOTAL Ca = 12·7 mgs.%: Ca IN 5 cc. SERUM = 0·635 mgs.: CO_2 TENSION = 42·56 mm. Hg.

·635	·580	·312	·362	64·8	F
·635	·571	·343	·363	60·1	G
·635	·550	·367	·380	62·0	H
·635	·550	·367	·387	64·0	H

DOG VI. TOTAL Ca = 12·6 mgs.%: Ca IN 5 cc. SERUM = 0·630 mgs.: CO_2 TENSION = 50·0 mm. Hg.

·630	·590	·312	·332	55·9	F
·630	·589	·343	·352	36·8	G
·630	·621	·367	·372	59·3	H
·630	·599	·367	·367	57·8	H

DOG VII. TOTAL Ca = 9·0 mgs.% Ca IN 5 cc. SERUM = 0·450 mgs.: CO_2 TENSION = 49·4 mm. Hg.

·450	·470	·312	·312	69·3	F
·450	·481	·367	·342	70·4	H

PARTIALLY THYRO-PARATHYROIDECTOMIZED DOGS.

TABLE VI.

DOG I. TOTAL Ca = 6·75 mgs.%: Ca IN 5 cc. SERUM = 0·337 mgs.: CO_2 TENSION = 50·0 mm. Hg.

Ca IN mgs. IN 5 cc. SERUM		Ca IN mgs. IN 5 cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·337	·340	·213	·250	80·0	B
·337	·368	·257	·291	96·4	D
·337	·363	·312	·296	83·1	F
·337	·368	·367	·352	100·0	H

DOG II TOTAL Ca = 6·13 mgs.%: Ca IN 5 cc. SERUM = 0·306 mgs.: CO_2 TENSION = 47·83 mm. Hg.

·306	·335	·257	·302	103·5	D
·306	·302	·257	·272	93·2	D
·306	·352	·312	·393	90·0	F
·306	·291	·406	·372	109·0	J

DOG III TOTAL Ca = 8·2 mgs.%: Ca IN 5 cc. SERUM = 0·410 mgs.: CO_2 TENSION = 48·64 mm. Hg.

·410	·410	·257	·281	84·5	D
·410	·391	·367	·392	101·6	H
·410	·391	·406	·412	101·9	J

COMPLETELY THYRO-PARATHYROIDECTOMIZED DOGS.

TABLE VII

DOG I. TOTAL Ca = 5·0 mgs.%: Ca IN 5cc. SERUM = 0·250 mgs.: CO_2 TENSION = 49·4 mm. Hg.

JULY 21. OPERATION. JULY 22. HYPERPNŒA OF CHEYNE-STOKES TYPE, WITH SALIVATION, FINE TREMOR OF MUSCLES, TETANY NOT SEVERE.

JULY 22. 70 cc. OF BLOOD DRAWN FROM FEMORAL ARTERY.

Ca IN mgs. IN 5cc. SERUM		Ca IN mgs IN 5cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·250	·314	·312	·280	99·2	F
·250	·246	·257	·221	90·0	D
·250	·310	·367	·312	102·8	H
·250	·291	·406	·332	95·2	J

DOG II. JULY 20. COMPLETE OPERATION AT NOON. JULY 21. 2 P.M., TREMORS OF NECK AND SHOULDER MUSCLES, INCREASED RESPIRATION AWITH SLIGHT SALIVATION. 8.P.M., MARKED TREMORS OF MUSCLES OF THE NECK AND OF THE HIND LIMBS. RESPIRATIONS VERY RAPID AND OF CHEYNE-STOKES TYPE. 9 P.M., 75 cc. OF BLOOD DRAWN ASEPTICALLY FROM THE FEMORAL ARTERY UNDER ANÆSTHESIA. 10 P.M., ANIMAL MUCH IMPROVED; ABLE TO STAND AND ABLE TO DRINK ON ACCOUNT OF REDUCED RESPIRATORY RATE: ANIMAL MENTALLY ALERT.

TOTAL Ca = 6·09 mgs.%: Ca IN 5cc. SERUM = 0·305 mgs.: CO_2 TENSION = 41·8 mm. Hg.

·305	·279	·257	·272	69·0	D
·305	·273	·312	·291	90·0	F
·305	·273	·367	·322	90·3	H

JULY 22. DOG IN MARKED TREMOR, CAPABLE OF STANDING BUT LEGS STIFF: RESPIRATION VERY IRREGULAR AND RAPID WITH A RATE OF OVER 200 PER MINUTE: 70 cc. OF BLOOD TAKEN ASEPTICALLY UNDER ETHER: RESPIRATION RATE NORMAL UNDER ETHER: DEFINITE IMPROVEMENT FOR 36 HOURS AFTER WITHDRAWAL OF BLOOD, THE DOG BECOMING QUITE LIVELY AND RESPONSIVE. TREMORS COMMENCED WITHIN 36 HOURS, BEING FELT OVER THE SHOULDER GIRDLE: RESPIRATION RATE AGAIN RAPIDLY RISING FROM 60 PER MINUTE TO 180 PER MINUTE.

TOTAL Ca = 5·1 mgs.%: Ca IN 5cc. SERUM = 0·255 mgs.: CO_2 TENSION = 44·84 mm. Hg.

·255	·287	·312	·261	82·0	F
·255	·299	·406	·322	93·4	J

DOG III. TOTAL THYRO-PARATHYROIDECTOMY PERFORMED JULY 21: DOG DIED IN THE NIGHT ABOUT 20 HRS. AFTER OPERATION, HAVING DEVELOPED HYPERPNŒA AND SLIGHT TREMOR DURING THE EVENING OF THE 21$^{\text{ST}}$: UNABLE TO OBTAIN FROM VEINS BECAUSE OF CLOTTING.

DOG IV. JULY 20: TOTAL THYRO-PARATHYROIDECTOMY.

JULY 26: THIS DOG HAS SHOWN NO SIGNS OF TETANY.

TOTAL Ca = 8·3 mgs.%: Ca IN 5cc. SERUM = 0·415 mgs.: CO_2 TENSION = 51·68 mm. Hg.

·415	·358	·367	·332	72·4	H
·415	·425	·257	·262	64·4	D

AUGUST 5: NO SIGNS OF TETANY HAVE DEVELOPED. TOTAL CALCIUM = 7·5 mgs.%.

DOG V. JULY 22: TOTAL THYRO-PARATHYROIDECTOMY PERFORMED: DOG IN EXCELLENT CONDITION.

JULY 23: SLIGHT HYPERPNŒA, MUSCULAR TWITCHING, AND SALIVATION DEVELOPING.

JULY 24: ALL THE MUSCLES OF THE ABDOMEN, SHOULDER, HEAD AND NECK AND LEGS IN CONSTANT AND RAPID TWITCHING. DOG IS VERY THIRSTY BUT UNABLE TO DRINK BECAUSE OF THE VERY RAPID RESPIRATION RATE AMOUNTING 240 PER MINUTE: THE DOG IS ABLE TO STAND, IS CONSTANTLY ON THE MOVE AND CONSTANTLY ATTEMPTING TO DRINK.

JULY 24: 10 A.M. BECAUSE OF THE SEVERITY OF THE CONDITION 70 cc. OF BLOOD WERE DRAWN FROM THE FEMORAL ARTERY. AT 2 P.M. THE CONDITION HAD GREATLY IMPROVED. ALL TREMORS HAD GONE AND RESPIRATION WERE ALMOST NORMAL.

JULY 26: NOON: TETANY ATTACK DEVELOPING WITH RAPID RESPIRATIONS. SALIVATION, MUSCLE TREMORS AND SPASTICITY OF LIMBS.

JULY 27: 9 A.M. SEVERE TETANY HAS DEVELOPED WITH STRONG SPASMODIC CONTRACTIONS OF MUSCLES OF THE HEAD AND JAW, WITH SHARP SNAPPING OF THE TEETH TOGETHER, CAUSING THE TONGUE TO BE BITTEN. RESPIRATION RATE, 250 PER MINUTE: SALIVATION AND THIRST MARKED: ANIMAL LYING SOAKED IN PERSPIRATION.

11 A.M. BECAUSE OF THE DISTRESSING CONDITION ANIMAL BLED TO DEATH.

COMPLETELY THYRO-PARATHYROIDECTOMIZED DOGS.

TABLE VII (CONT).

DOG V. (CONT.)

JULY 24: TOTAL Ca = 5·0 mgs. %: Ca IN 5cc. SERUM = 0·250 mgs.: CO_2 TENSION = 47·12 mm Hg.

Ca IN mgs. IN 5 cc. SERUM		Ca IN mgs. IN 5cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·250	·313	·257	·254	100·4	D
·250	·313	·312	·282	100·8	F

JULY 26: TOTAL Ca = 4·7 mgs. %: Ca IN 5cc. SERUM = 0·235 mgs.: CO_2 TENSION = 46·36 mm. Hg.

·235	·256	·257	·246	100·0	D
·235	·268	·312	·271	97·8	C

JULY 27: TOTAL Ca = 4·7 mgs. %: Ca IN 5cc. SERUM = 0·235 mgs.: CO_2 TENSION = 42·56 mm. Hg.

·235	·305	·312	·227	96·6	F
·235	·332	·367	·300	99·1	H

DOG VI. *JULY 22: TOTAL THYRO-PARATHYROIDECTOMY PERFORMED.*

JULY 30: USUAL SIGNS OF TETANY; 70 cc. OF BLOOD TAKEN FROM FEMORAL ARTERY FOR ESTIMATIONS AND ANIMAL BLED TO DEATH.

TOTAL Ca = 4·6 mgs. %: Ca IN 5cc. SERUM = 0·230 mgs.: CO_2 TENSION = 50·16 mm. Hg.

·230	—	·312	·263	93·1	F
·230	·322	·367	·284	87·4	H

DOG VII. *JULY 29: TOTAL THYRO-PARATHYROIDECTOMY PERFORMED: VERY DEFINITE SIGNS OF TETANY DEVELOPED WITHIN 48 HOURS.*

TOTAL Ca = 5·2 mgs. %: Ca IN 5cc SERUM = 0·260 mgs.: CO_2 TENSION = 50·16 mm. Hg.

·260	·269	·257	·251	94·3	D
·260	·307	·367	·312	98·8	H

DOG VIII. *AUG. 2: NOON. TOTAL THYRO-PARATHYROIDECTOMY PERFORMED.*

AUG. 3: 9 A.M. MARKED TREMOR IN MUSCLES OF THE HEAD AND NECK, SHOULDERS, ABDOMEN. RESPIRATION RATE, 220 PER MINUTE: 70 cc. OF BLOOD TAKEN FROM FEMORAL ARTERY FOR ESTIMATIONS.

AUG. 4: CONDITION RELIEVED TEMPORARILY BY WITHDRAWAL OF 70 cc. OF BLOOD.

AUG. 5: 2 P.M. TREMORS OF MUSCLES VERY MARKED: PERSPIRATION AND SALIVATION MARKED: AT 7 P.M. TREMORS OF MUSCLES AS AT 2 P.M.: HÆMORRHAGE FROM THE TONGUE DUE TO INJURY FROM SPASMODIC CONTRACTION OF JAW MUSCLES: PERSPIRATION PROFUSE: SPASTICITY OF LIMBS MARKED: ANIMAL UNABLE TO STAND: CONDITION VERY SEVERE.

AUG. 3: TOTAL Ca = 4·6 mgs. %: Ca IN 5cc. SERUM = 0·230 mgs.: CO_2 TENSION = 50·16 mm. Hg.

·230	·286	·312	·241	73·5	F
·230	·290	·312	·240	73·0	F

AUG. 4: TOTAL Ca = 6·0 mgs. %: Ca IN 5cc. SERUM = 0·304 mgs.: CO_2 TENSION = 51·88 mm. Hg.

·230	·310	·312	·302	97·6	F
·230	·309	·367	·383	98·5	H

AUG 5: TOTAL Ca = 5·4 mgs. %: Ca IN 5cc. SERUM = 0·270 mgs.: CO_2 TENSION = 49·56 mm Hg.

·270	·179	0	·120	98·8	NO COMPENSATION FLUID USED
·270	·138	0	·126	93·3	

THE PERCENTAGE OF CALCIUM WHICH IS DIFFUSIBLE CALCIUM IS CALCULATED AS FOLLOWS:

$$\text{DIFFUSIBLE CALCIUM} = \frac{\text{Ca IN DIALYZATE AFTER DIALYSIS} \times 2 \text{ MINUS Ca ADDED.}}{\text{ORIGINAL CALCIUM IN SERUM.}}$$

It appears, however, that the rapid loss of diffusible calcium from the blood demands a correspondingly rapid liberation of calcium from its colloidal state.

The great demand of the organism for calcium one can understand upon witnessing the severity of the condition. It seems feasible, therefore, to postulate that there is an attempt on the part of the organism to supply the bivalent salts necessary for the maintenance of a normal condition of irritability of the nervous system, and the method adopted is that of a rapid liberation in ionic form of all the colloidal calcium in the blood. Unfortunately, in virtue of its free state in the circulation, the calcium is very readily lost by way of the kidneys, which are unable to respond properly to the abnormal condition by setting up a mechanism of curative adaptation. Under these conditions it is evident that once the store of colloidal calcium in the blood is utilised there must be a call upon the calcium of the tissues. That there is a muscle-protein breakdown in tetany has been amply proved by several workers, most notably by Paton, Findlay and Burns (1914–15), and Paton and Findlay (1916). It is possible, therefore, that there is a continual breakdown of muscle-protein, which is responsible for the production of a toxic substance, and it is this steady decomposition of protein which is the mechanism whereby an attempt is made to meet the call of the nervous and other tissues for calcium. In view of the urgent needs of the organism, and probably as a result of the protein decomposition, the calcium is thrown out in that form in which it is most easily utilised.

In considering the reason for the presence of diffusible calcium in the blood in tetany, the somewhat contradictory facts that both hydrochloric acid and calcium salts are effectual in the treatment of tetany open up a suggestion that the increase in the H-ion concentration of the blood during acute tetany may be a means whereby more calcium can be liberated from protein combination. The lessened alkalinity of the blood would favour the dissolving of the calcium, and would thereby make it immediately available for the body as soon as it was broken from its protein combination.

It must not be forgotten that the alteration in calcium metabolism is a sign of tetany, and that the causative factor is probably a body associated with the creatine of muscle protein.

The signs of parathyroid tetany.—In all these dogs, with the single exception of No. IV, definite symptoms of tetany developed. It is evident from the figures for Dog IV that there was no derangement of the free and combined calcium balance, but it will be noted that there is a slight though definite loss in total calcium.

Dogs II, V, VI, VII and VIII developed signs of tetany within thirty-six hours, and it will be seen from the notes in the tables that the attacks were, with only two exceptions, of a severe type. None of these animals suffering from severe tetany survived for more than six days. The signs noted as definitely indicating parathyroid tetany were the increased respiratory rate of from 200 to 250 per minute, with noisy expiration, often of a Cheyne-Stokes type, the profuse salivation and nasal secretion, all of which developed within forty-eight hours. The characteristic muscle tremors were invariably in evidence within twenty-four hours as fine muscle twitchings which could be felt most clearly over the shoulder girdle. These tremors became more and more marked until, in some cases by the end of the second day after operation, they had become coarse spasmodic twitchings. These were markedly in evidence in Dogs V, VII and VIII, in which cases the spasmodic contractions of the temporal and masseter muscles were undoubtedly distressing, the latter causing injury to the tongue by the sudden snapping together of the teeth.

The spasticity of the limbs, on account of which the animal is forced to lie on its side, the inability to stand, the cardiac irregularities, vomiting, loss of appetite, and at times profuse perspiration, were all in evidence two days, at the latest three days after operation.

The mild or more chronic types of tetany described by other observers in which the animals lived for two or three weeks after operation have not been seen throughout this investigation. All of ours were definite cases of tetany, most of them severe, terminating fatally within one week. The severity of these attacks was always greatly ameliorated by the withdrawal of the 70 c.c. of blood for the necessary examinations.

The severe condition occasioned by complete removal of the thyro-parathyroid apparatus may well account for the results recorded.

SUMMARY.

(1) The calcium content of 100 c.c. of normal blood amounts to the following average figures: Total 9·12 mg., plasma 8·11 mg., cells 1·10 mg. In tetany these drop to the average figures: whole blood 5·7 mg., plasma 5·26, and cells 0·46. These figures show a loss of calcium amounting to 27·2 per cent. for whole blood, to 54·4 per cent. for cells, and to 35·2 per cent. for the plasma.

(2) Diffusible calcium in normal serum averages from 60 to 70 per cent., while in severe parathyroid tetany it amounts to 94 per cent. of the total calcium.

(3) While there is an immediate state of alkalosis following parathyroidectomy, this condition is not necessarily marked, and with the development of the signs of severe tetany it passes rapidly into a condition of acidosis as shown by a steadily falling pH of the blood.

(4) The immediate relief of the condition consequent upon the withdrawal of 70–100 c.c. of blood is indicative of a toxic causative factor.

(5) The calcium deficiency and the great loss of colloidal calcium are probably indicative of a rapid protein disintegration.

REFERENCES.

Cushny, A. R.—(1920) *J. Physiol.*, **53**, 391.

Halverson, J. O., and Bergheim, O.—(1917) *J. Biol. Chem.*, **29**, 337.

Henderson, Y., and Morris, W. H.—(1917) *J. Physiol.*, **31**, 217.

Levy, R. L., Rowntree, L. G., and Marriott, W. McK.—(1915) *Arch. Int. Med.*, **16**, 389.

Lyman, H.—(1917) *J. Biol. Chem.*, **29**, 169.

Paton, D. N., Findlay, L., and Burns, D.—(1914–15) *J. Physiol.*, **49**, 17.

Paton, D. N., Findlay, L.—(1916) *Quart. J. Exper. Physiol*, **10**, 203.

Von Mysenbug, L., Pappenheimer, A. M., Zucker, T. F., and Murray, M. F.—(1916) *J. Biol. Chem.*, **47**, 529.

THE EXPRESSION OF THE RESULTS OF THE WASSERMANN REACTION IN UNITS.

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It has become the fashion nowadays to express biological reactions in units where the exact amount of the reacting substance cannot be determined gravimetrically. The advantage of such a procedure for recording the strength of the Wassermann reaction is at once apparent for the purpose of comparison with other laboratories and for noting the effects of treatment.

At present the Wassermann reaction is usually expressed in such nomenclature as negative, weak positive, positive, strong positive, etc., or as some numeral which indicates the degree of reaction in a particular dilution of the patient's serum. For instance, in the Fildes-McIntosh method (1913, 1918) the results are recorded by the figures 4, 3, 2, 1 or 0; 4 meaning a complete inhibition of hæmolysis; 3, 25 per cent. of hæmolysis; 2, 50 per cent.; 1, 75 per cent., and 0 complete hæmolysis. In the actual test 5 falling dilutions of patient's serum, 0·1 to 0·006 c.c., are used, so that if the reaction changes, say, from 44200 to 42000, we can appreciate that the amount of Wassermann substance is decreasing, but we do not know the amount of the decrease. Browning and Kennaway (1920) a short time ago suggested a criterion of positivity based upon a comparison of the amount of complement giving complete lysis with a serum and with the negative control. In France, Vernes' syphilimetric method (1920) is another attempt to obtain an interpretation of the serum reaction, which will give a better control of treatment.

The adoption of a unit system for the Wassermann reaction is not quite as easy as would appear, because it is not sufficient merely to multiply the serum dilution by some simple index. Such a procedure as this does not take into account the fact that in the Wassermann test different degrees of positivity may be obtained in the same tube, *i. e.* different degrees of hæmolysis. So that a unit system for the Wassermann must, in addition, take into account this possible variation in hæmolysis as well as the variation in the amount of serum.

The constant on which we have fixed for our unit system is the antigen. Previous researches have shown that the McIntosh-Fildes antigen (1913) is constant in its complement-fixing properties. But in addition to the antigen there are two other constituents of the Wassermann reaction which may be regarded as constants in that they are standardised before each test: (*a*) sensitised red cells, and (*b*) 2½ minimal hæmolytic doses of complement. The exact value of these constants can be best shown by examining each separately.

The antigen, as has already been indicated, shows no variation either in regard to specific fixation or inhibitory power, and so must be regarded as a constant. The sensitised red corpuscles are used solely as indicators of the presence of free complement. Slight variations in their susceptibility to hæmolysis and to the extent to which they are packed by the centrifuge does occur, but it must be small. To get the best results the red cells should be as fresh as possible, as keeping for a few days in the ice chest, and more especially if some preservative has been added, renders them more or less sensitive to hæmolysis Very occasionally red cells are obtained which show an increased resistance to hæmolysis. Such variations, however, will always be shown up in the preliminary standardisation. Lastly in the test proper the value of the 2½ minimal hæmolytic doses of complement is undoubtedly a constant, particularly in relation to the 0·5 c.c. of sensitised red cells. The preliminary standardisation of complement determines its hæmolytic titre, and, in addition, insures that it does not behave in an abnormal manner in the presence of antigen, while any abnormal inhibition on the part of the serum to be tested will be shown up by the control tube which contains 1¼ M.H.D. of complement.

It has been stated that so variable are the results of Wassermann tests on the same serum that the reaction can be of little value for comparative tests, but the constancy of the Wassermann reaction based on the principles we have adopted (McIntosh, 1918) is greater than the experience of certain writers would allow. Provided the preliminary standardisation is accurately carried out constant results should follow. The results given below were obtained by testing the same pooled serum over a period of five months. The serum was placed in small vaccine bulbs and kept at −10° C. The results were judged by opacity in the ordinary routine method without any attempt at estimation of the hæmolysis by means of a colour scale.

25 . 1 . 23	4 4 4 4 3 1 0
1 . 2 . 23	4 4 4 4 4 1 0
8 . 2 . 23	4 4 4 4 1 0 0
8 . 3 . 23	4 4 4 4 2 0 0
15 . 3 . 23	4 4 4 4 3 0 0
31 . 5 . 23	4 4 4 4 1 – –

As already pointed out, it is the different degrees of specific adsorption in any single dilution of the serum as shown by degrees of hæmolysis corresponding to the figures 4, 3, 2 or 1 which complicates matters. As a preliminary to an elaboration of a unit system it was necessary to determine the specific fixation of the complement which corresponded to these degrees of hæmolysis from an estimation of the amount lost through nonspecific adsorption by antigen and by serum.

In order to do this a double set of experiments were set up to determine—

(1) Extent to which complement was fixed by antigen alone.

(2) Extent to which complement was fixed in the presence of antigen with a negative serum.

In each of the above a series of 10 tubes was taken. In the end tube was placed 2½ M.H.D. of complement, and in the rest diminishing quantities down to one-tenth that amount, the actual quantities being 0·25 c.c. to 0·025 c.c.

To the first series was then added 0·25 c.c. of Wassermann antigen and then saline to bring the total volume to 0·5 c.c.; to the second series 0·25 c.c. of antigen + 0·05 c.c. of a negative human serum, the volume as before being brought up to 0·5 c.c.

Each series was incubated for 1 hour at 37°C., and at the end of that time 0·25 c.c. of sensitised red cells were added and the tubes placed in the water-bath for 20 minutes.

Results were read as percentage of hæmolysis by diluting each tube with saline, allowing to settle, and comparing with a standard scale prepared from the same blood.

Thus—

0 per cent.	=	Wassermann	4
25 ,,	=	,,	3
50 ,,	=	,,	2
75 ,,	=	,,	1
100 ,,	=	,,	0

Table I gives a composite chart determined from these two series of experiments. It is compiled from 8 antigen tests and 20 negative sera tests. The actual hæmolytic titre of the complement as estimated in the ordinary preliminary Wassermann standardisation is given in brackets at the side.

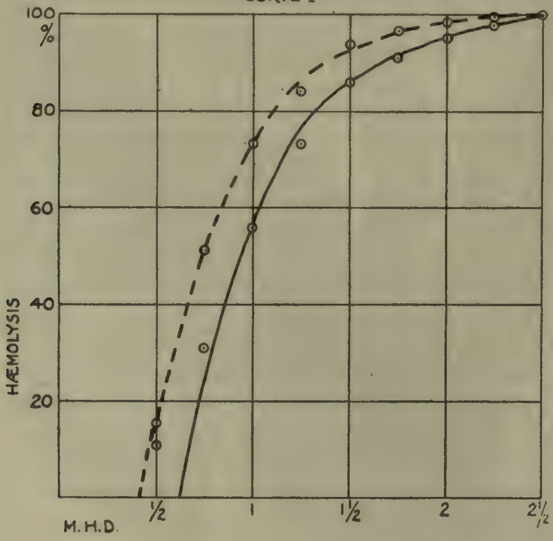
Curve I shows graphically these results and the effects of negative sera on the non-specific adsorption of complement. The full line represents the adsorption by antigen in the presence of negative sera, and the dotted line without serum. The amounts of complement corresponding to the different degrees of hæmolysis or readings in the *first tube* of the Wassermann can readily be determined, while the actual

TABLE I

TUBES

		1	2	3	4	5	6	7	8	9	10	
ANTIGEN	1	0	22	52	70	85	92	95	95	99	100	1/24
	2	0	15	57	85	90	96·5	97	98·5	100	100	1/28
	3	0	15	65	?90	?95	94	97·5	100	100	100	1/30
	4	0	10	30	65	77	92·5	96·5	100	100	100	1/16
TOTALS		0	62	204	220	252	375·0	386	393·5	399	100	
AVERAGE		0	15·5	57	73	84	93·7	96·5	98·4	99·7	100	
ANTIGEN & SERUM	1	0	9	26	61	74	90	92	97	99·5	100	
	2	0	10	30	63	74	87	90	94	97	99·3	
	3	0	24	49	67	77	85	90	93	96	99	
	4	0	3	20	43	69	83	91	98	100	100	
TOTALS		0	46	125	234	294	345	363	382	392·5	398	
AVERAGE		0	11	31	56	73	86	91	95	98	99·5	

CURVE I



amounts of complement (in units of M.H.D.), which must be fixed specifically to give these readings, is obtained by subtraction from 2½ M.H.D.—the amount of complement used in the test.

TABLE II.

Wassermann test-reading.	Hæmolysis in percentage.	Specific adsorption of complement in M.H.D. units.
4	0	1·87
3	25	1·75
2	50	1·57
1	75	1·25
0	100	0·0

In the second and succeeding tubes the quantities of complement adsorbed will not be the same for similar readings of hæmolysis because the quantity of serum is decreasing. This, however, is easily calculated, and is shown on Table III.

TABLE III

W. R. READING	QUANTITY OF SERUM IN TUBE IN C.C.S	M. H. D COMPLEMENT TO BE FIXED	RECIPROCAL OF Q. OF SERUM × DOSES OF COMPLEMENT FIXED = NEW WASSERMANN UNITS
44444	1/160	2·057	329·1
44443		1·937	309·9
44442		1·757	281·1
44441		1·437	229·9
44440	1/80	2·045	163·6
44430		1·925	154·0
44420		1·745	139·6
44410		1·425	114·0
44400	1/40	2·02	80·8
44300		1·9	76·0
44200		1·72	68·8
44100		1·4	56·0
44000	1/20	1·97	39·4
43000		1·85	37·0
42000		1·67	33·4
41000		1·35	27·0
40000	1/10	1·87	18·7
30000		1·75	17·5
20000		1·57	15·7
10000		1·25	12·5

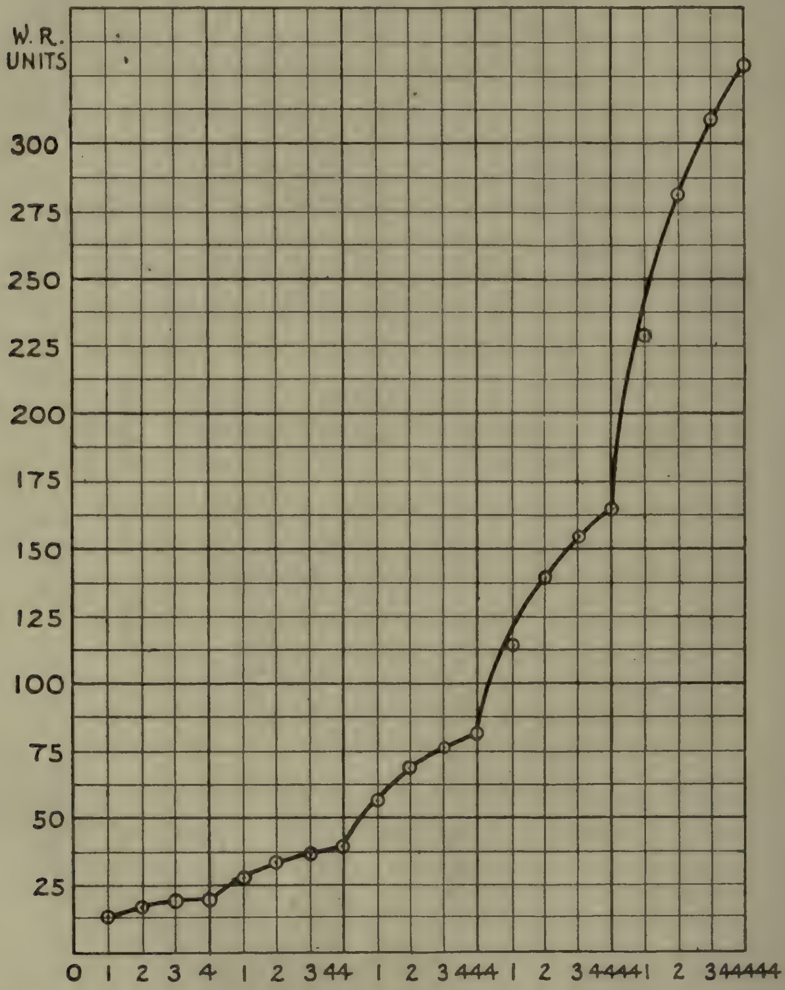
In all these calculations it is necessary to assume that the non-specific adsorption of a positive serum is identical with that of a negative serum.

Contrary to a general belief, negative sera, therefore, are not protective, but 0·1 c.c. adsorbs about 0·2 of an M.H.D. of complement.

In the second tube of the Wassermann test we have only 0·05 of serum, which will adsorb, not 0·2, but 0·1 M.H.D. Hence for this tube, in estimating the M.H.D. fixed, we must add 0·1 of an M.H.D. to all the figures of column 3 of Table II. A similar correction must be applied for the remaining tubes.

As a suitable unit for the Wassermann reaction we propose the quantity of complement in M.H.D. fixed (specific fixation) multiplied by the reciprocal of the fraction of a cubic centimetre of serum used in the last tube showing inhibition. The relation between such units and the old readings of the Wassermann is indicated in Curve II. From this diagram the unit value can be seen at a glance.

CURVE II.



It now remains to set a standard of positivity for the new units. It is usual to take a 3 reading in the first tube as indicative of syphilitic infection in a case without definite history. This in the unit system is equivalent to 17·5 units (*i.e.* complement fixed for 25 per cent. hæmolysis = 1·75 M.H.D. × reciprocal of fraction of c.c. of serum employed $= \dfrac{1}{\frac{1}{10}} = 10$ ∴ units = 1·75 × 10 = 17·5).

In a treated case, on the other hand, even a "1" is regarded as indicative of some remaining infection, and this figure is represented by 1·3 × 10 = 13 units.

Curve II is based on this relationship, and from it the new unit value for any reading of the Wassermann can be determined directly.

The advantages of the unit system have already been commented on, particularly with regard to obtaining comparable results between different laboratories and obtaining a better indication of the effects of treatment. The application of the unit system would not interfere with the particular Wassermann technique used at particular laboratories. It would only mean that a standard serum of so many units strength and a standard antigen would have to be supplied. The method, however, can be applied directly to any method of carrying out the Wassermann reaction by reducing the number of M.H.D. of complement fixed to the required M.H.D. for a 5 per cent. suspension of sheep cells. The result from the laboratory would be given as positive, weak positive, negative, etc., with the units given in brackets at the side.

COMPARISON WITH THE UNIT OF THE SIGMA TEST OF DREYER AND WARD.

Quite recently we had an opportunity of comparing this proposed Wassermann unit with the unit system employed in the Sigma test of Dreyer and Ward (1921). A series of bloods were under examination on behalf of the Medical Research Council for the purpose of comparing the Wassermann and Sigma tests, and which will shortly be published.

Table IV gives an analysis of these results; on the left the ordinary Wassermann readings are given and on the right the Sigma results which corresponded. Plotting these results, W.R. units against Sigma units, a good line can be obtained when the Sigma averages are compared with the new

TABLE IV

W. R. READING	NEW UNITS	GROUP AVERAGE	AVERAGE OF SIGMA READINGS	GROUP AVERAGE OF SIGMA READINGS
44444	329 1	273·6		87
44443	309·9		128	
44442	281·1		53	
44441	229·9		82	
44440	163·6	142·8	46	45·2
44430	154 0		64	
44420	139·6		35	
44410	114·0		36	
44400	80 8	70·4	37	26·4
44300	76 0		29·5	
44200	68·8		19	
44100	56·0		20	
44000	39·4	34·2	3·6	9·3
43000	37·0		10·2	
42000	33·4		10·0	
41000	27·0		8·3	
40000	18·7	16·1	5·3	7·08
30000	17·5		9·9	
20000	15·7		7·4	
10000	12·5		5·3	

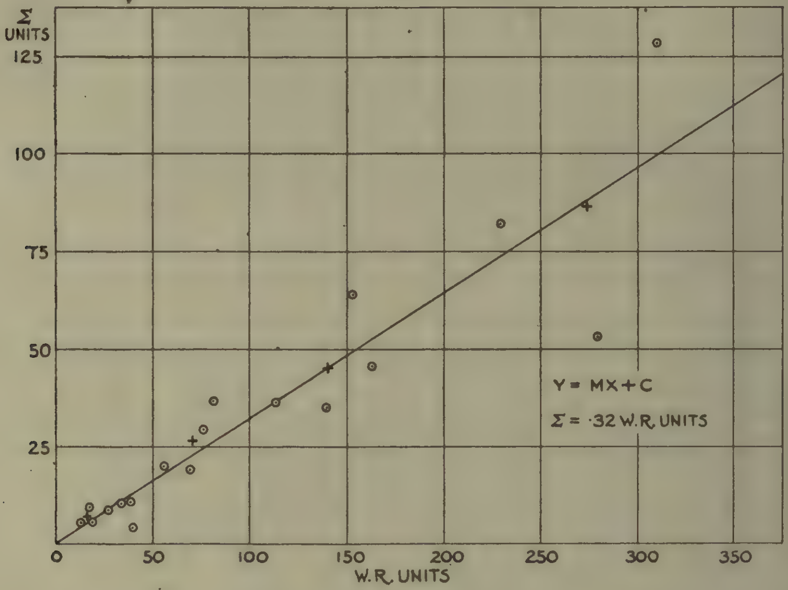
Wassermann units (Curve III). A still better line, as perhaps is to be expected, is obtained if we plot out the group average of these units, these groups corresponding to the five tubes of the Wassermann reaction.

Solving this curve from the formula—

$$Y = MX + C$$

we find find that Sigma unit = 0·317 W.R. units.

CURVE III.



Dreyer and Ward take as the diagnostic limit 1·63 units, which, according to the above formula, = 5·05 W.R. units.

Again, a W.R. "3," regarded as diagnostic, = 19 W.R. units or 19 × 0·317 = 6·3 Sigma units.

This shows that there is a discrepancy in the setting adopted as standard in these two tests. Either that of the Wassermann is too high or that of the Sigma too low.

The Wassermann, however, has now had many years of experience in diagnosis and in treatment, and the standard is built up on this basis. In the light of these researches the Wassermann standard may want some adjustment. However, clinical experience, particularly in watching the effects of treatment, and time can alone determine what the standard should be. Because the Sigma obtains slightly "better" results in cases under treatment, it has been assumed by certain workers that it is a more delicate test. This is not necessarily so, and, as the above figures show, the improvement probably depends on the basis adopted as the standard of specificity. Further, the close parallelism of the systems, as shown by the curves, suggests that the Wassermann reaction and syphilitic precipitation tests are produced by one and the same substance in spite of experiments to the contrary (Mackie, 1923).

CONCLUSIONS.

A unit system for the expression of Wassermann results is described above. The method is applicable to any technique of carrying out the Wassermann test by reducing the number of M.H.D. of complement fixed to the required M.H.D. for a 5 per cent. suspension of sheep cells, and multiplying this result by the reciprocal of the quantity in cubic centimetres of serum employed.

REFERENCES.

Browning, C. H., and Kennaway, E. L.—(1920) *J. Hyg.*, **19**, 87.
Dreyer, G., and Ward, H. K.—(1921) *Lancet*, **1**, 956.
Fildes, P., and McIntosh, J.—(1913) *Brain*, **36**, 193.—(1918) *Med. Res. Council Spec. Report Series*, No. **14**, p. 32.
McIntosh, J. (1918) *Lancet*, **1**, 630.
Idem., and Fildes, P.—(1913) *Ztschr. f. Chemother.*, **1**, 79.
Mackie, T. J.—(1923) *J. Path. & Bact.*, **26**, 120.
Vernes, A.—(1920) 'Atlas de Syphilimetrie,' Paris.

AUTO-DISSOCIATION OF AGGLUTININ-ANTIGEN COMPLEX.

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The compound formed between antigen and antibody has hitherto been supposed to be a fairly stable one, at any rate by those holding Ehrlich's views. A certain amount of work has been done in efforts to resolve this compound, so that the antibody may be procured free, so far as possible, from the other constituents of the serum from which it was obtained.

Notably, Huntoon (1921) has attempted to effect this release and recovery with some success; he used methods of salting out combined with alternations of freezing and warming and recovered a portion of his immune bodies.

The following property of the compound formed, which I discovered by following up an apparent error in technique, is, I believe, new to the literature.

Recently I have been testing the staphylococcal group for serological types and found it necessary to use absorption of agglutinin tests. The method was the usual one, consisting in absorption of a 1:25 dilution of serum by a suspension of 50,000 millions per 1 c.c. of the coccus to be tested with the usual controls. After incubating for two hours at 37° C. and centrifuging out the coccus, the supernatant serum was tested for remaining agglutinins against the homologous coccus.

On one occasion I was interrupted in a test at the point when the incubation had been completed, leaving the separation of the cocci to be done, and I therefore placed the cocci, still in contact with the diluted serum, to stand till the following day at laboratory temperature, as I had occasionally done before. I was prevented from returning to complete the test for some 72 hours, and when I then had separated the cocci and serum and finished the test I was surprised to find that none of the cocci, which included two homologous examples as controls together with six for testing, had absorbed any appreciable amount of agglutinin. I naturally thought that I had made some error and immediately repeated the test with the same materials, but taking only 24 hours over it, to find the homologous cocci, with two of those for testing, had absorbed the agglutinin completely, while four others had not done so at all.

On reviewing the previous unsatisfactory experiment I failed to find any flaw in the technique and determined to repeat it, allowing a pause of 72 hours to intervene between absorption and separation as before. The result was the same as in the former one. I repeated this with other sera and cocci of different types and always the agglutinins were found free at the end, the original titre of the serum being hardly altered, if at all.

Obviously the connection of antigen and agglutinin appeared to be of an evanescent nature under the conditions I was using, but the dissociation did not take place within 48 hours of absorption. Evaporation did not cause it, as the tubes were tightly sealed, and lost only a very small fraction, if anything, during the waiting period. The menstruum used throughout was normal saline (0·85 per cent. NaCl, alcohol purified, in glass-distilled water).

The following experiment was then put up to settle the point:

A quantity of 1 in 25 dilution of a serum (No. 46) was made, and suspensions of the homologous No. 46 coccus of 50,000 millions and 100,000 millions per 1 c.c. These two strengths were used in case it might be thought that the smaller dose, though hitherto apparently sufficient, was not in reality large enough to absorb all agglutinin. Half of the serum dilution was mixed with an equal quantity of the lower strength suspension and the other half with the same quantity of the higher one. After incubation at 37° C. for two hours and a thorough shaking, one third of each mixture was put aside to wait for 96 hours at laboratory temperature, while the remaining portions were centrifuged at high speed till the cocci were completely sedimented, to eyesight. The fluids were then carefully removed and half of each were tested immediately in the usual manner, while the other halves were put to stand by the side of the serum and coccal mixtures for 96 hours. The protocol (see Table I) shows the result.

It will be seen that the agglutinin has been completely absorbed by both strengths of coccal suspension in the portion tested immediately, but it is otherwise in the portions left to stand for 96 hours in contact with the cocci. The serum in this case has received back its agglutinin to its full strength apparently. The serum, on the other hand, which was separated from the coccus, as might be expected, still showed the absence of agglutinins, though it is true there was a trace shown at the 1 in 100 dilution, with the weaker dose of cocci. These traces are difficult to account for, and may be due to a variety of causes. I think the explanation in this case probably is that it is very difficult to remove the last cocci from a suspension, and although apparently clear the fluid may still contain nearly 100 million cocci per 1 c.c. These cocci, should they still remain in the fluid, might well give up their fraction of agglutinin on standing, hence possibly the " Trace."

TABLE I.

Absorbing dose. No. 46 coccus.	Unabsorbed serum. 1 in—				Absorbed serum. 1 in—				Remarks.
	100	200	400	800	100	200	400	800	
Immediate test.									
50,000 mill.	+ +	+ +	+	(+)	—	—	—	—	Complete absorption of agglutinins.
100,000 mill.					—	—	—	—	
Tested after 96 hours' wait.									
50,000 mill.	+ +	+ +	+	(+)	tr.	—	—	—	Test of serum, separated from cocci, after 96 hours' wait. Almost complete absorption.
100,000 mill.					—	—	—	—	
50,000 mill.	+ +	+ +	+	(+)	+ +	+ +	+	(+)	Left in contact with cocci. No absorption.
100,000 mill.					+ +	+ +	+	(+)	

+ + = complete agglutination. + = obvious agglutination, fluid not clear. (+) = agglutination only visible with × 8 lens.

The result of this experiment, I think, shows irrefutably that agglutinin, after absorption, dissociates itself from its antigen after standing at normal temperature for a sufficient time.

The next experiment was to ascertain whether dissociation would occur in normal saline alone. No agglutination, however, occurred after allowing the absorbing cocci to stand in saline for 96 hours, the cocci having previously been well washed free of serum.

A similar experiment to the last was now tried, substituting normal rabbit serum (serum, both normal and immune, was always from the rabbit). This showed no agglutination previously with the same coccus used for the experiment with plain saline. The result was not so good as with the immune serum, for although there was some agglutinin in the serum when tested after its 96 hours' contact with the absorbing coccus, the titre barely amounted to half that of the original immune serum. Partial dissociation, however, had occurred.

The next experiment was undertaken to see whether agglutinin could be transferred by this method from one immune serum to another, though heterologous, immune serum.

Two serologically different cocci were taken with their homologous sera—No. 20, a Type I *St. pyogenes*, and No. 46, a Type II *St. epidermidis albus*. The normal agglutinations of these were as follows:

	1 in 100	200	400	800
No. 20 serum *v.* No. 20 coccus	+ +	+ +	+	+
No. 20 ,, *v.* No. 46 ,,	—	—	—	—
No. 46 ,, *v.* No. 46 ,,	+ +	+	+	(+)
No. 46 ,, *v.* No. 20 ,,	(+)	—	—	—

Both cocci gave no agglutination with 1 : 100 normal serum.

A dilution of No. 46 serum was put up in the usual manner to absorb with No. 46 coccus. The coccus, then, after centrifugalisation and well washing twice in saline, was added to a dilution of No. 20 serum (heterologous). This mixture and the centrifuged off No. 46 serum were then put to stand at room temperature for 96 hours, and then, after centrifuging the No. 46 coccus from the No. 20 serum, tested in the usual way for both agglutinins.

	Serum dilution. 1 in— 100	200	400	800
No. 20 serum after 96 hours *v.* No. 20 coccus	+	+	+	+
No. 20 ,, ,, *v.* No. 46 ,,	+ +	+	+	+
No. 46 ,, (absorbed) *v.* No. 46 ,,	+	(+)	—	—
No. 46 ,, ,, *v.* No. 20 ,,	+	(+)	—	—

It will be observed that the No. 46 serum which was absorbed by its homologous coccus is very much reduced in strength for its own coccus, but all agglutinin is not quite absorbed, probably for the reason given above. For No. 20 coccus, however, it is also raised in titre to a small extent, the explanation of which I am not able to suggest.

The No. 20 serum, however, which has been in contact with the absorbing coccus for 96 hours, has lost a small fraction of its agglutinin for its own coccus, but has gained agglutinin for No. 46 coccus, which was entirely absent before, almost up to the full titre of the No. 46 serum.

The agglutinin therefore seems to be transferable from one immune serum to another in the manner shown.

REFERENCE.

Huntoon, F. M.—(1921) *J. Immunol.*, **6**, 117.

THE COMPLEMENT-FIXATION TEST IN THE DIAGNOSIS OF GONOCOCCAL INFECTION IN WOMEN.

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THE method adopted in carrying out the test was essentially that advocated by L. W. Harrison. Two types of antigen were used, one made up following exactly the technique of D. Thomson (1918), and another prepared by a method described below, parallel observations being made with each. In some cases, also, parallel tests were made with the gonococcal antigen prepared by D. Thomson and put on the market by Genatosan, Limited.

A powerfully deviating serum was used to compare the strength of different antigens, and in all deviation tests as a positive control.

Tests were made on sera taken from 172 cases attending the women's V.D. clinics of the Elizabeth Garrett Anderson and the Royal Free Hospitals, care being taken to include as far as possible all stages and manifestations of the disease.

METHODS.

Preparation of a Strongly Deviating Serum.

A rabbit was injected at weekly intervals with increased doses of a gonococcal emulsion made from 24-hour cultures of eight different strains of gonococci, grown on a serum-agar medium of pH 7·9.

After a dose of 6000 million gonococci had been reached a serum was obtained which in a 1/1500 dilution deviated three doses of complement completely, and in a 1/6400 dilution gave agglutination with an emulsion made from those strains of organisms which had been used for immunisation.

Preparation of Antigens.

The method used was a modification of that described by Altmann and Schultz (1909), with reference to the typhoid bacillus. A standard emulsion of gonococci—made from 24-hour cultures of eight different strains—was made, such that 1 c.c. contained 3000 million organisms. An equal volume of 4 per cent. commercial antiformin was added and the resulting solution heated to 56° C. for 45 minutes. At the end of this time a perfectly clear solution was obtained, in which absence of free chlorine was proved by testing with starch-iodide paper.

The solution was titrated back to neutral with decinormal acid, the point taken as "neutral" being that at which no colour was given with phenolphthalein and a definite purple tint with bromo-cresol-purple (*i. e.* between pH 6·8 and pH 8·3). Normal saline containing 0·5 per cent. carbolic acid was added till the final volume was three times that of the original emulsion. The resulting solution, containing 1000 million dissolved gonococci per c.c., was used as a stock antigen. This will be referred to as Antigen A.

A second antigen was made from the same eight strains, carrying out in every detail the technique described by Thomson. This, which also contains 1000 million of dissolved gonococci per c.c., will be referred to as Antigen B.

Technique Employed in Carrying Out the Test.

1. Inactivation and dilution of the serum to be tested: Inactivation was performed by heating in a water-bath at 55° C. for 15 minutes.

Experiments made with human sera, in dilutions of 1/2, 1/5 and 1/10, showed that the 1/2 dilution, after inactivation, was liable to deviate complement too frequently for its use to be practicable. 1/5 was found to be the lowest dilution in which the serum was not anticomplementary; this solution with 0·85 per cent. saline was therefore used habitually.

2. Titration of complement against red cells in the presence of antigen: To 8 Wassermann tubes was added 0·1 c.c. of fresh guinea-pig serum (complement) in the following dilutions:—

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1/10	1/15	1/20	1/30	1/40	1/60	1/80	1/120

To each was added 0·1 c.c. of the stock antigen diluted 1/5 in the case of Antigen A, 1/10 for Antigen B.

To each again was added 0·1 c.c. of normal saline, to make the volume the same as that in the test itself. As control a second series of tubes was put up, containing complement diluted as before, to each being added 0·2 c.c. of saline. In this way any anticomplementary action on the part of the antigen itself could be detected.

The tubes were shaken, placed in the ice-chest for one hour, and then transferred to the water-bath at 37° C. for half-an-hour. To each was then added 0·1 c.c. of a 2·5 per cent. suspension of sensitised sheep corpuscles. The tubes were then replaced in the water-bath for 15 minutes and the reading taken at the end of that time.

The greatest dilution of complement producing complete hæmolysis in the series containing antigen was taken as the minimum hæmolytic dose (M.H.D.), this factor being determined before every experiment. The M.H.D. in the series without antigen was generally found to be less than this; a complement working at 1/30 with antigen would hæmolyse at 1/40 with saline. Any antigen deviating complement to a greater degree than this was rejected as too anticomplementary for use.

3. The test itself: For each serum to be tested, four tubes (I, II, III and IV) were used.

To each of the four was added 0·1 c.c. of the inactivated serum diluted 1/5.

To I was added 0·1 c.c. of complement, containing 1 M.H.D.
To II ,, ,, ,, ,, 1 ,,
To III ,, ,, ,, ,, 2 ,,
To IV ,, ,, ,, ,, 3 ,,

To II, III and IV were added 0·1 c.c. of antigen diluted 1/5 for Antigen A, or 1/10 for Antigen B. To I was added instead 0·1 c.c. of saline, this tube acting as a serum control. Parallel with tubes II, III and IV were put up three tubes containing antigen and complement as in II, III and IV, but in which the serum was omitted and replaced by 0·1 c.c. of saline; these formed the antigen control.

The tubes were placed in the ice-chest overnight. Next morning 0·1 c.c. of a 2·5 per cent. suspension of sensitised sheep-cells was added, and the reading taken after 15 minutes in the water-bath at 37° C.

The serum control tube I and the antigen control tubes should show complete hæmolysis. Known positive and negative sera were always included in a series of tests, and the results of an experiment were accepted only if all the controls were satisfactory.

EXPERIMENTS TO ESTIMATE AND COMPARE THE PROPERTIES OF ANTIGENS.

1. *The greatest strength in which the antigens could be used* was determined by titrating a constant volume of each antigen in dilutions of 1/2, 1/5 and 1/10, against varying dilutions of complement, after the method already described. It was found that both antigens in a 1/2 dilution were strongly anticomplementary.

Antigen A diluted 1/5 was only slightly anticomplementary, permitting hæmolysis with a complement dilution of 1/30, the complement used alone giving a M.H.D. of 1/40.

Antigen B diluted 1/5 gave with this same complement a M.H.D. of 1/10, and was therefore rejected in this strength.

In a 1/10 dilution it deviated complement only to the same extent as the 1/5 dilution of Antigen A.

Antigen A could therefore be used at double the strength found possible for Antigen B.

The gonococcus antigen supplied by Genatosan Limited was found to be identical with Antigen B as regards its anticomplementary action.

2. *Comparison of the antigenic power of antigens.*—Each antigen in its appropriate dilution was put up with a series of increasing dilutions of the antigonococcal rabbit serum, 3 M.H.D. of complement being added in each case.

It was found that, using Antigen A 1/5, complete deviation was given by the serum in a 1/1500 dilution. With Antigen A 1/10, the highest dilution of serum giving complete deviation was 1/900; with Antigen B 1/10 partial deviation was given by the same dilution of serum.

Using the commercial antigen, no deviation was obtained in a serum dilution of more than 1/200; this, however, may well be explained by the fact that the commercial antigen was non-specific to the serum, whereas Antigens A and B were both made from the strains used in preparation of the serum.

3. *Effect on antigens of titrating them to varying degrees of hydrogen-ion concentration.*—A series of antigens was made as previously described, but the final reaction was titrated to a definite pH, using phenol-red as indicator, and matching to standard phosphate solutions of known pH.

It was found that both Antigens A and B at pH 7·0, 7·4 and 7·8 showed no appreciable difference as regards either their anticomplementary or antigenic properties. When brought to a pH as alkaline as 8·2 both became slightly more anticomplementary. It seemed therefore that in the rough titration to "neutral" of the standard antigens, the variations of pH possible within the range of experimental error were of negligible importance.

4. *Anticomplementary effect of too prolonged fixation at low temperatures.*—Griffith and Scott (1920) recommend that a serum to be tested should be left in contact with complement and antigen for some hours at 0° C. before the sensitised red cells are added, claiming that a more thorough fixation of complement is obtained by this means.

It was noticed on several occasions that the antigen controls treated in this way became anticomplementary to doses of complement with which, in the preliminary titrations, they had allowed complete hæmolysis to occur. A series of tubes was therefore put into the ice-chest, each containing the same amount of antigen with one M.H.D. of complement, a tube being withdrawn and tested with sensitised red cells at 2-hourly intervals. It was found that complete hæmolysis was obtained up to the fifteenth hour. After 16 hours there was slight deviation of complement; at 18 hours deviation was complete.

In performing the test, therefore, 15 hours was the time allowed for fixation in the ice-chest, with satisfactory results.

RESULT OF 172 EXPERIMENTS ON SERA TAKEN FROM PATIENTS ATTENDING A WOMEN'S V.D. CLINIC.

The following charts give the results of the test with an analysis of the cases examined.

TABLE I.

	Total number examined.	Doses of complement deviated.						Deviation positive.		Valid positives (1 M.H D. or more).	
		1,2-1	1	2	3	4	5	Total.	%.	Total.	%.
I.—G. cases:											
1. Pathologically proved	35	13	12	2	—	1	—	28	80	15	42·8
2. Clinically typical	44	16	14	—	—	—	—	30	68	14	31·8
II.—Cases not suggestive of G., but giving a positive Wassermann reaction	52	14	5	—	—	—	—	19	36·5	5	9·6
III.—Negative control cases, suggestive of neither G. nor S.	41	3	—	—	—	—	—	3	7	0	0·0

TABLE II.

Analysis of the 79 G. Cases in Group I of Table I.

	Number examined.	Deviation positive.	Deviation. negative.
1. Acute urethritis or endocervicitis, *i. e.* within 4 weeks of onset	13	7	6
2. Chronic urethritis or endocervicitis	34	25	9*
3. Reinfection in old cases	6	6	0
4. Acute salpingitis	2	1	1
5. Chronic salpingitis	7	6	1
6. Bartholin's abscess	6	4	2
7. Vulvo-vaginitis of children	6	5	1
8. Arthritis at some stage of the disease	5	4	1

* Four of these cases were regarded as clinically cured.

TABLE III.

Comparison of Antigens A and B in 55 Cases giving Deviation with Each.

	Slight hæmolysis.	About 50 per cent. hæmolysis.	Nearly complete.	Complete to 1 M.H.D.
Antigen A	4	13	12	26
Antigen B	6	18	10	21

It will be seen that, of the 79 cases regarded as genuine gonococcal infections, 58 (73 per cent.) gave a positive result. Of these, however, 55 deviated no more than 1 M.H.D. of complement; 26 completely and 29 partially, using Antigen A; and 21 completely and 34 partially, using Antigen B. (See Table III.)

Of the remaining three cases, two deviated 2 M.H.D. complement completely; one showed complete deviation on two occasions with 4 M.H.D. Details of these three cases are given :

Case "1826." History of acute urethritis about 6 months previous to the taking of test. Bartholini abscess opened 6 weeks ago. Attack of acute salpingitis 3 weeks ago from which patient is recovering. Gonococcus grown in culture and isolated from both urethra and cervix. Deviation given to 2 M.H.D. (complete).

Case "1765." Acute urethritis and endocervicitis 5 weeks before test taken. Gonococcus grown in culture and isolated from urethra. Deviation to 2 M.H.D. (complete).

Case "1680." Chronic urethritis and endocervicitis of 18 months' duration. Arthritis affecting both knee-joints in the first months after infection. Gonococcus found repeatedly in film and culture. Deviation to 4 M.H.D. (complete).

In the attempt to obtain a higher deviation, a small number of sera were tested against their specific antigens, the gonococcus isolated from the patient being in each case made up to the same strength as the stock antigens. It was found that case "1765," which deviated 2 M.H.D. with the stock antigen, now deviated 4 M.H.D. Of five other cases, each giving complete deviation to 1 M.H.D. with the stock antigen, two now deviated 3 M.H.D., two deviated 2 M.H.D., and one remained unchanged in its deviating power.

Reference to the chart will show that the large number of positive sera in Group II deviated in every case 1 M.H.D. or less of complement. Here, too, the antigens gave very comparable results, Antigen B frequently giving a partial deviation where Antigen A gave complete deviation, but neither failing to show in some degree a deviation given by the other.

The three non-gonococcal and non-syphilitic cases entered as positive gave partial deviation only to 1 M.H.D. of complement.

CONCLUSIONS.

The results obtained establish the fact that a certain degree of deviation is produced in the majority—73 per cent.—of cases regarded as gonococcal, deviation being most constantly shown, as might be expected, in those cases where infection is of some weeks' standing, and particularly where the disease has passed beyond the purely local stage.

The low degree of deviation obtained in all but three of the series seems, however, to be a serious drawback to the adoption of the test as a means of diagnosis. The margin left for experimental error must necessarily be small in dealing with such fine shades of deviation as that to a fraction of a single dose of complement.

Moreover, the fact that a high percentage of presumably non-gonococcal cases give positive results with this reaction and with the Wassermann reaction introduces another large factor of possible error.

It seems, however, that the complement-deviation test for the gonococcus may be of definite value, in cases where the Wassermann reaction is negative, *if deviation to less than 1 M.H.D. is disregarded.* As shown by Table I, rejection of smaller degrees of deviation would eliminate the errors of the negative control cases in Group III, and would reduce that of the syphilitic cases of Group II from 36 per cent. to 10 per cent., leaving 37 per cent. of positive cases in Group I. *The deviation to at least one complete dose given by this residue can be regarded as a definite entity reasonably free from error, and should be taken as the minimum in which confidence can be placed in using the test for diagnostic purposes.*

It should be remembered that there is no room for experimental error in working with deviations within such narrow limits ; that the technique should be adhered to rigidly in every detail, and the carrying out of the test kept in the hands of experienced workers. *If these precautions are observed, a positive result may be taken as a definite aid in diagnosis.*

As regards an antigen for general adoption, the Antigen A made by the antiformin method here described has given consistently in these experiments slightly the more satisfactory results. It is not difficult to prepare, and it has shown no deterioration after being kept for three months at a temperature of 0°C.

I wish to thank Dr. Ledingham for facilities given me for work in the Bacteriological Department of the Lister Institute, and for his helpful criticism. Dr. Elizabeth Lepper has given me much invaluable advice throughout. Dr. Rorke and Dr. Balmer, by giving me access to the patients attending their clinics, have made possible the taking of histories and the collection of material for the work.

REFERENCES.

ALTMANN, K., AND SCHULTZ, J. H.—(1909) "Verwendung von Bakterien-Antiformin-extracten als Antigene bei der Komplementbindung," *Ztschr. f. Immunitätsforsch.* (Orig.), **3**, 98.

GRIFFITH, F., AND SCOTT, W. M.—(1920) 'The Complement Fixation Test in Syphilis, commonly known as the Wassermann Test,' Ministry of Health; Reports on Public Health and Medical Subjects, No. 1, London, H.M. Stationery Office.

THOMSON, D.—(1918) "A New Method of Preparing Gonococcus Antigen, and a New Technique for the Complement Deviation Test," *Lancet*, **2**, 42.

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VARIATION PHENOMENA IN STREPTOCOCCI: FURTHER STUDIES ON VIRULENCE AND IMMUNITY.

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In a previous communication (Cowan, 1922) it has been stated that from individual strains of streptococci two types of organisms can be "dissociated"; these have been called "rough" (R) and "smooth" (S) types. The former grows as a sediment in broth and shows dense colonies with irregular outline on agar, while the organisms are large and appear in bunches or chains; the latter render broth turbid and form more translucent colonies with regular outline on agar, while the individual cocci are smaller and show fewer bunches and chains. The most important difference found was, however, that of virulence, the "S" type being markedly more virulent than the "R" type.

In the following communication are recorded further experiments designed to elucidate more precisely the characteristics and relationships of these two types of organisms.

VIRULENCE.

It had been found that the "S" type of streptococcus was much more virulent than the "R" type when the organisms were injected intraperitoneally and subcutaneously into mice and when given intravenously to rabbits. The following experiments were performed in order to determine whether the same difference could be elicited when they were given to mice intravenously.

Exp. 1.—$\frac{1}{4}$ c.c. of a 24-hour serum broth culture of the "R" and "S" types of each of 7 strains of hæmolytic streptococci was given intravenously to mice—the tail vein being used. In each case 2 mice were used, so that 14 mice received the "R" type, and 14 mice received the "S" type of organism.

Result.—At the end of 6 days, 9 mice given "R" type and 12 mice given "S" type were dead.

Exp. 2.—$\frac{1}{20}$ of a 24-hour growth on a serum agar slope of the same "R" and "S" types used in Exp. 1 was injected intravenously into mice, in duplicate.

Result.—At the end of 7 days, 10 of the 14 mice receiving "R" and all (14) of the mice receiving "S" type were dead.

Exp. 3 was a repetition of Exp. 2, except that $\frac{1}{25}$ of the growth from a serum agar slope was used in place of $\frac{1}{20}$.

Result.—At the end of 8 days, 5 of the 14 mice receiving the " R " type and 10 of the 14 mice receiving " S " type were dead.

(Death of a mouse more than 8 days after injection was not considered as due to the injection.)

It would seem from these experiments that if large doses of "R" and "S" types of streptococci are given intravenously to mice, the difference in the mortality, although evident, is not marked. When the dose decreases, however, the difference becomes more prominent.

IMMUNITY TO THE "S" TYPE CONFERRED BY INOCULATION WITH THE "R" TYPE.

A. *Homologous Protection.*

An indication had been obtained (Cowan, 1922) that a dose of the live " R " type would protect a mouse from a lethal dose of the " S " type administered later. The following experiments were performed with a view to verifying and amplifying this finding.

Exp. 1.—" R " type of strain " B.C. " was grown on serum agar slopes and an emulsion made in normal saline. Doses of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ of a slope were injected intraperitoneally into mice in duplicate, so that 12 mice received "R" organisms. At the end of 14 days 10 of the 12 survived, and these and one control were each given intraperitoneally 1 m.l.d. of the " S " type of strain " B.C."

Result.—The control and 3 treated mice died overnight while the remaining treated mice were alive and well in 9 days. The treated mice all showed abscesses in the peritoneal cavity.

Exp. 2.—6 mice were each given intraperitoneally $\frac{1}{6}$ of the 24-hour growth from a serum agar slope of the " R " type of strain " B.C." Of these, 4 were alive in 8 days, and each with 1 control was given intraperitoneally 1 m.l.d. of the " S " type of strain " B.C."

Result.—The control mouse died overnight while the treated mice were all alive and well in 9 days.

These experiments were repeated with strains " Ludwig " and " 2716." Table I gives the number of mice which received the " R " type, those surviving this, and those which survived a later m.l.d. of the homologous " S " type—that is, those with an immunity produced by the " R " type against the " S " type.

TABLE I.

Strain.	Number of mice given "R" type.	Number of mice surviving and given " S " type.	Number of mice surviving and apparently immune to " S " type.
" B.C. "	18	14	11
" 2716 "	18	11	8
"Ludwig"	23	16	8
	59	41	27

Thus 27 of 41 mice had acquired an immunity against the virulent "S" type by a previous intraperitoneal injection of the homologous "R" type.

B. *Cross Protection.*

The mice which had received the "R" type of streptococci and later survived a m.l.d. of the corresponding "S" type were given a m.l.d. of the "S" type of another strain. A considerable number of these survived, indicating that one strain produced an immunity against another. The following experiments were performed in order to determine whether this immunity was due to the primary dose of "R" organisms.

Exp. 1.—6 mice were each given intraperitoneally $\frac{1}{6}$ of the growth from a serum agar slope of the "R" type of strain "B.C." Eight days later 4 were given intraperitoneally the "S" type of strain "Ludwig" and two were given the "S" type of strain "2716": controls were inoculated with each strain.

Result.—All controls died overnight.

One given strain "2716" died on injection and the other survived. 1 given strain "Ludwig" died in three days and 1 in 4 days, while 2 survived. All the treated mice showed abscesses in the peritoneal cavity.

The experiment was repeated with the "R" type of strains "2716" and "Ludwig." Table II gives the number of mice used in each case and the number surviving.

TABLE II.

Strain.	Number of mice given "R" type.	Number given "S" type of strain "B.C."		Number given "S" type of strain "2716."		Number given "S" type of strain "Ludwig."	
		Died.	Survived.	Died.	Survived.	Died.	Survived.
"B.C."	6	—	—	1	1	2	2
"2716"	4	0	2	0	0	2	0
"Ludwig"	4	0	2	2	0	—	—
	—						
	14 : 7 died ; 7 survived.						

There is evidently some cross-protection of mice whichever "R" type is given and later the "S" type of another strain; but the number of mice inoculated with each strain and the number of strains used is too small to draw any conclusion as to a relationship of different strains with respect to production of cross-protection.

"LOCAL" OR "GENERAL" CHARACTER OF PROTECTIVE MECHANISM.

It seemed possible that the immunity conferred on mice by the "R" type of streptococcus against the "S" type might be a local one, as, in each case, the two types had been given intra-peritoneally. In order to obtain some evidence for or against this possibility, experiments were carried out in which the "R" type of organisms were given intra-peritoneally and the "S" type intravenously and *vice versâ.*

Exp. 1.—6 mice were each given $\frac{1}{6}$ of the growth from a 27-hour-old serum agar slope of "R" type of strain "B.C.," which was given intra-peritoneally. In 8 days all were alive, and these and 3 controls were given 1 m.l.d. of "S" type of strain "B.C." intravenously.

Result.—4 treated mice died almost immediately, some with convulsions. The controls and 2 remaining treated mice died within 24 hours. All the treated mice showed abscesses in the peritoneal cavity.

Exp. 2.—Repetition of Exp. 1.

Result.—Again 4 of the 6 treated mice died almost immediately after injection of the "S" type; some had convulsions. One died in 2 days and 1 was alive and well in 10 days.

The almost instantaneous deaths of these treated mice, when given the "S" type of organisms intravenously, pointed to anaphylaxis as a possible cause. To overcome this difficulty the experiment was repeated, but a desensitising dose was given intra-peritoneally before the intravenous dose.

Exp. 3.—6 mice were each given intra-peritoneally $\frac{1}{6}$ of the growth from a serum agar slope of the "R" type of strain "B.C." After 7 days they were each given a very small dose of the same type intra-peritoneally. The next day they were given 1 m.l.d. of the "S" type intravenously.

Result.—The control mouse died in 4 days and all the treated ones survived. 1 of the treated mice when killed after 12 days had pus in the joints of one foot from which a hæmolytic streptococcus was recovered.

Exp. 4.—Repetition of Exp. 3 with the strain "Ludwig."

Result.—All the treated mice and the controls died within 24 hours after receiving the "S" type intravenously. None died immediately after injection and the treated mice did not show abscesses in the peritoneal cavity.

Exp. 5.—Repetition of Exp. 3 with the strain "2716," 4 mice being used.

Result.—1 treated mouse died immediately after injection of the "S" type, but had no convulsions; 1 died in 24 hours; the control died in 3 days; the remaining 2 treated mice survived.

Exp. 6.—6 mice were each given intravenously $\frac{1}{20}$ of the 24-hour growth from a serum agar slope of the "R" type of strain "B.C." After 14 days all were alive, and each and 1 control was given intraperitoneally 1 m.l.d. of the "S" type of strain "B.C."

Result.—4 treated mice and the control died overnight; 1 treated mouse died in 2 days and 1 in 8 days.

Exp. 7.—Repetition of Exp. 6. 4 mice survived the intravenous dose of "R" type, and these and 1 control were given intra-peritoneally 1 m.l.d. of "S" type of strain "B.C."

Result.—All the mice died within 24 hours.

Of the experiments detailed above, No. 3 and No. 5 indicate that the immunity against the "S" type of streptococcus induced by a previous intra-peritoneal injection of the "R" type is not local only. The fact that the mice treated with the "R" type of strain "Ludwig" (Exp. 4) had apparently no immunity may be in some way connected with the fact that no abscesses were found in the peritoneal cavity.

In Experiments 6–7 the dose of the "R" type of organisms was very small, and its failure to give definite immunity against the "S" type was to be expected.

PROTECTION INDUCED IN MICE BY HEAT-KILLED ORGANISMS.

Exp. 1.—6 mice were each given, intra-peritoneally, $\frac{1}{8}$ of the growth from a serum agar slope of the "R" type of strain "B.C.," the suspension having been heated to 56° C. for 1 hour. After 10 days each of these mice and a control was given intra-peritoneally 1 m.l.d. of the "S" type of the same strain.

Result.—The control died within 24 hours. 2 treated mice died in 2 days and 2 in 3 days, while 2 survived.

None of the treated mice which died showed abscesses, but the survivors when killed in 10 days had definite abscesses in the peritoneal cavity.

Exp. 2.—Repetition of Exp. 1 with the preliminary injection of killed "S" type of organisms in place of killed "R" type.

Result.—The control died within 24 hours after the injection of the test m.l.d. of living "S" type; 4 treated mice died in 2 days while 2 survived. The treated mice which died showed no abscesses, but the survivors when killed had abscesses in the peritoneal cavity.

There is evidently some protection acquired by mice against the "S" type of streptococcus, by previous injection of killed organisms, of both the "R" and "S" type. Here, too, there may be some association of protection with abscess formation.

EXAMINATION OF ABSCESSES FOUND IN THE PERITONEAL CAVITY OF MICE AFTER INJECTION OF "R" TYPE OF STREPTOCOCCUS.

A number of mice were each given, intra-peritoneally, 0·5 c.c. of a 24-hour serum broth culture of the "R" type of strain "B.C." These were killed in 3, 5, 7, 10 and 17 days and the abscesses found in the peritoneal cavities were examined histologically.

In all cases there was a definite localisation of the inflammatory process. The 3- and 5-day abscesses were in most part made up of masses of organisms and polymorphonuclear leucocytes. In the older ones the number of small round-cells increased and the number of organisms decreased. In the 10-day-old abscesses fibroblasts could be distinguished at the margin of the abscesses, and in 17-day specimens there was active proliferation of the fibroblasts around and into the abscess. This condition was found in both large and small abscesses although more marked in the smaller ones.

Cultures made from abscesses found in mice from 3 days to 2 months after the intra-peritoneal injection of "R" type of streptococcus have always given a growth of hæmolytic streptococcus of the "R" type.

DISCUSSION.

The results here recorded of the effect of intravenous injection into mice of the "R" and "S" types of streptococcus confirm the findings previously reported, that there is a definite relationship between virulence and the "S" type of organism in a strain of streptococcus.

There is also no doubt that the "R" type when given intra-peritoneally to mice excites the production of immunity against the "S" type. From the fact that mice are protected against the "S" type when given either intravenously or intra-peritoneally, it would appear that the immunity is of a general character, and is not confined to the peritoneal cavity where the "R" type of organisms was given, and where, in most cases, they persisted for some time in localised abscesses. There would appear to be some association between the occurrence of abscesses and the acquisition of immunity, for mice which proved immune invariably showed abscesses in the peritoneal cavity. A minority only of mice treated and found not to be immune showed these abscesses. It is probable that the abscesses found were due to the initial dose of "R" organisms and not to the test dose of "S" organisms, as they yielded "R" cultures and as the "R" type does form abscesses in the majority of cases; the "R" type of strain "B.C." has never failed to do so if given in such doses as used in these experiments.

Protection of mice against the "S" type of heterologous strains of streptococci is apparently given by injection of the "R" type into the peritoneal cavity. The experiments recorded show some difference with respect to the immunity produced by one "R" type against two other "S" types, but the number of strains used and the number of mice in each series is too small to afford any clue as to whether a grouping of the hæmolytic streptococci may be made on such a basis. They do, however, raise the question of that possibility.

It is proposed to repeat on a larger scale those experiments which have indicated a general immunity as opposed to a local immunity and also those dealing with cross-protection, in order to compare the numbers of mice so protected with the numbers given in Table I of this paper. Also, it will be of interest to determine, over a large series of mice, whether preliminary doses of heat-killed "R" or "S" types are as effective as the living "R" type in producing an immunity against the living virulent "S" type.

SUMMARY.

(1) The "S" type of streptococcus is more virulent for mice than the "R" type when both are given intravenously.

(2) An injection of "R" type organisms gives considerable protection to mice against a subsequent lethal dose of the "S" type even when the test dose is administered by another route. The immunity is, therefore, not purely local, nor is it confined to the "S" type of the homologous strain of the immunising "R" type.

(3) Heat-killed "R" and "S" types in some cases produce immunity in mice against the "S" type.

(4) The "R" type of organisms, when injected intra-peritoneally into mice, localise in abscesses which tend to heal by fibrosis.

(5) There would appear to be an association of immunity with the presence of these localised abscesses.

I wish to thank Prof. Ledingham for much helpful advice and criticism in the course of this work.

REFERENCE.

COWAN, MARY L.—(1922) "Variation Phenomena in Streptococci, with Special Reference to Colony Form, Hæmolysin-Production and Virulence," *Brit. J. Exper. Pathol.*, **3**, 187.

THE SPLEEN AND IMMUNITY REACTIONS TO JENSEN'S RAT SARCOMA.

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In view of the fact that in man the post-mortem room rarely reveals cancerous metastases in the spleen (although, according to Kettle (1912–13), microscopic nodules are more common in that organ than might be supposed), it was decided to investigate the question in the case of the rat and Jensen's rat sarcoma.

Previous work had shown us that when simultaneous grafts of the same sample of tumour are made into numerous organs of the same animal growth occurs less frequently, and the resulting mass of tumour is smaller in the spleen than in most other tissues. Thus in experiments upon 36 rats, the mean amounts of tumour found at death in the various organs and the frequency of growth in them were as follows:

Mean Amount of Tumour in cubic millimetres.

Sub-cutaneous.	Intra-peritoneal.	Kidney.	Liver.	Testicle.	Spleen.	Brain.
1071	1675	902	604	470	333	22

Percentage Frequency of Definite Growth.

Sub-cutaneous.	Intra-peritoneal.	Kidney.	Liver.	Testicle.	Spleen.	Brain.
72%	61%	67%	58%	60%	50%	<50%

To test the question, series of rats were inoculated intra-splenically or subcutaneously with portions of a rapidly growing Jensen's rat sarcoma either in the form of fresh tumour or in that of material altered by repeated freezing and thawing or by exposure to a heavy dose of X-rays, and the effect of this procedure upon development of a subcutaneous graft or established tumour was considered. The degree of effect in each series of rats was estimated by (1) the amount of tumour present at the site of the subcutaneous test inoculation on the 10th day, and (2) the ultimate survival of healthy tumour-free rats. Each step of the experiment was associated with numerous controls.

EXPERIMENTS USING FRESH TUMOUR GRAFTS THROUGHOUT.

1. *The Effects of Intra-splenic or Subcutaneous Immunisation upon the Development of a Subcutaneous Graft.*

Twenty normal rats were inoculated into the right flank and 20 into the spleen with grafts of a rapidly growing Jensen's rat sarcoma, and seven days later the survivors received grafts into the left flank.

On the 10th day after the second (test) inoculation the condition in the left flanks of the rats was as follows:

	Number of rats showing tumours.	Mean volume of tumour.	
		Per rat of series.	Per cent. of control.
Intra-splenic immunisation	5 (30%)	49 c.mm.	26
Subcutaneous immunisation	13 (65%)	103 ,,	56

The numbers of healthy and tumour-free rats at the conclusion of the experiment, 8 weeks after immunisation and 7 weeks after the test subcutaneous inoculation are given in Table I.

The experiment was repeated, and on the 10th day after the second (test) inoculation the condition in the left flanks of the rats was as follows:

	Number of rats showing tumours.	Mean volume of tumour.	
		Per rat of series.	Per cent. of control.
Intra-splenic immunisation	5 (25%)	35 c.mm.	11
Subcutaneous immunisation	4 (20%)	76 ,,	25

The numbers of healthy and tumour-free rats at the conclusion of the experiment, 5 weeks after the immunising and 4 weeks after the test inoculation are seen in Table I.

TABLE I.—*Numbers of Healthy and Tumour-free Animals at End of Experiment.*

	Intra-splenic immunisation.	Subcutaneous immunisation.
Exp. 1	3 rats (23%)	None.
Exp. 2	14 rats (74%)	2 rats (10%)

On the basis of the observed mortality of the controls after subcutaneous inoculation it was to be expected theoretically that none of the doubly inoculated rats in Exp. 1 (100% and 100% deaths from tumour) would survive, and in Exp. 2 (90% and 70% deaths from tumour) that 3% of them would survive.

From these experiments we conclude that a definite degree of immunity against subcutaneous inoculation is conferred by intra-splenic immunisation with fresh tumour. This immunity is greater than that conferred by subcutaneous immunisation.

2. *The Effects of Intra-splenic or Subcutaneous Inoculation with fresh Tumour Grafts upon an Established Subcutaneous Tumour.*

During the investigations upon which this and an earlier paper (Lazarus-Barlow and Parry, 1923) are based, three series of 20 rats each were inoculated subcutaneously with fresh tumour at a time when a 7 days' tumour was existent in the other flank. The course of the established growth was apparently unaltered. For this reason intra-splenic inoculation alone is considered here.

A number of normal rats received subcutaneous grafts on both sides of the body of a rapidly growing tumour. On the 8th day after inoculation 17 animals with definite subcutaneous nodules received intra-splenic grafts. Other 19 animals bearing tumours were similarly treated on the 13th day after subcutaneous inoculation. Measurements of the subcutaneous nodules in all animals were made at 2-day intervals.

The course of test (subcutaneous) tumour growth after the intra-splenic inoculation in the two series of animals is seen in Table II where are given the mean volumes of the experimental test tumours, expressed as percentages of the controls.

TABLE II.

8-*day Subcutaneous Tumour.*

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
119	89	80	78

13-*day Subcutaneous Tumour.*

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
80	75	86	88

Ultimately all the control and experimental animals died as the result of enormous tumour growth.

From these experiments it appears that the effects of the intra-splenic inoculation of fresh tumour upon an 8-day and a 13-day established and growing subcutaneous tumour are alike in kind but differ in degree. In both there is an immediate inhibition of rate of growth which causes the experimental tumour to lag behind the control. For an 8-day tumour this lag amounts to about 33%, and is still observable three weeks after the intra-splenic inoculation. For a 13-day tumour the lag is much smaller and lasts for less than a week. No evidence was obtained of a definite retrogression of growth in any instance, though the consistency of the experimental tumours appeared to be less, and deep as well as superficial flattening occurred in some cases.

EXPERIMENTS USING FOR PRELIMINARY INOCULATION GRAFTS OF TUMOUR THAT HAD BEEN REPEATEDLY FROZEN AND THAWED.

Inasmuch as fresh grafts when inoculated into spleen may undergo development into definite tumours (although this is less likely to occur than when a graft is subcutaneous), it was desirable to determine whether an immunity could be produced by the inoculation of material that was altered chemically as little as possible but nevertheless was incapable of multiplication. Previous experiments by Haaland (1910) had shown that living cells are necessary for the production of immunity when inoculation is subcutaneous, but the question is unsolved in respect of intra-splenic inoculation. Various means for attaining this end were tried, notably grinding tumour with sand, killing of cells with chloroform, repeated freezing and thawing. Ultimately the last-mentioned method was adopted, and since it was found that six times repeated freezing of a thin slice of a 100% (subcutaneous) tumour on the plate of an ether freezing microtome followed by rapid thawing prevented growth of the graft in 80% of the cases, eight times repeated freezing and thawing was used in the experiments to be described.*

(1) *The Effects of Intra-splenic or Subcutaneous Immunisation with Frozen and Thawed Tumour upon the Development of a Subcutaneous Graft of Fresh Tumour.*

Eighteen normal rats received intra-splenic and other twenty received subcutaneous grafts of a tumour which had been frozen and thawed eight times and yielded no trace of growth in any of the controls. Seven days after immunisation all the animals received in the left flank grafts of a fresh rapidly growing tumour.

On the 10th day after the test (subcutaneous) inoculation the condition in the left flank of the rats was as follows:

	Number of rats showing tumours.	Mean volume of tumour. Per rat of series.	Mean volume of tumour. Per cent. of control.
Intra-splenic immunisation	11 (65%)	134 c.mm.	55
Subcutaneous immunisation	16 (80%)	219 c.mm.	90

The number of healthy and tumour-free rats at the end of the experiment 5 weeks after the immunising and 4 weeks after the test inoculation are seen in Table III. In spite of the fact that all the subcutaneously-inoculated and 71% of the intra-splenically inoculated rats bore tumours at the end of the experiment, the mean size of these tumours was about half that obtaining in the controls.

TABLE III.—*Numbers of Healthy and Tumour-free Animals at End of Experiment.*

Intra-splenic immunisation.	Subcutaneous immunisation.
4 rats (29%)	None.

We conclude from these experiments that intra-splenic immunisation with repeatedly frozen and thawed tumour confers a certain degree of general resistance to later subcutaneous inoculation with fresh tumour. General resistance induced in this way is greater than that following subcutaneous immunisation. After intra-splenic or subcutaneous immunisation with repeatedly frozen and thawed material a subcutaneous graft of fresh tumour develops at a slower rate than the control.

(2) *The Effects of Intra-Splenic Inoculation of Frozen and Thawed Tumour upon an Established Subcutaneous Tumour.*

Eighteen rats were taken for each of these experiments. The frozen and thawed material used for intra-splenic inoculation in combination with 8-day tumours yielded no trace of growth in any of the controls; that used for intra-splenic inoculation with 13-day tumours yielded small growths after a long delay in 30%.

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* Use of liquid air would be more rapid but was not available; rapid thawing was carried out in the 60° C. paraffin oven.

The course of test (subcutaneous) tumour growth after the intra-splenic inoculation in the two series of animals is seen below in Table IV, where are given the mean volumes of the experimental test tumours expressed as percentages of the controls.

TABLE IV.

8-day Subcutaneous Tumour.

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
134	86	81	79

13-day Subcutaneous Tumour.

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
77	68	78	78

Ultimately all the control and experimental animals except one of the 8th-day series died as the result of enormous tumour growth. Hence no essential difference was observed between the action of fresh and of repeatedly frozen and thawed tumour material, when injected intra-splenically, upon the growth of an established and growing subcutaneous tumour. In both cases a delay in growth is produced but no retrogression occurs.

EXPERIMENTS USING FOR IMMUNISATION GRAFTS OF TUMOUR THAT HAD BEEN EXPOSED TO A HEAVY DOSE OF X-RAYS.

Chambers, Scott and Russ (1922), practising subcutaneous inoculation in all cases, have shown that protection against a subsequent inoculation is conferred when the material used for the preliminary grafts has been subjected to a "lethal" dose of X-rays under standard conditions. It appeared desirable to apply the method in the case of intra-splenic inoculations also. Batches of 20 normal rats were therefore inoculated intra-splenically with Jensen's rat sarcoma that had been exposed for 55 minutes and 66 minutes respectively to X-rays under standard conditions.* Subcutaneous controls and intra-splenic and subcutaneous controls with non-radiated material were made at the same time. A week later all the rats were inoculated subcutaneously with grafts of a rapidly-growing tumour.

So far as concerns the effects of subcutaneous immunisation with heavily-irradiated material we confirm the observations of Chambers, Scott and Russ, for 42% of the rats immunised with 55 minutes' irradiated material and 60% of those immunised with 66 minutes' irradiated material developed no growth, whereas over 97% of the controls died from tumour.

In the case of intra-splenic immunisation with irradiated material the result of our experiments was not so clearly defined. Under these conditions a larger proportion of the animals developed growths at the site of test subcutaneous inoculation than was the case when non-radiated material was used for immunisation. There was evidence also from the volume of the subcutaneous tumour on the 10th day after test-inoculation that growth was more rapid when the material used for intra-splenic immunisation had been irradiated. Thus the mean volume of the test tumour on the 10th day after inoculation was, in the 55 minutes' series, 3 times, and in the 66 minutes' series nearly 6 times that which obtained in the corresponding series in which the immunising grafts had not been exposed to X-rays. At the end of the experiment, in the 55 minutes' series 40% of the rats (controls 0) and in the 66 minutes' series 42% (controls 25%) were healthy and tumour-free, while in the corresponding series in which the immunising grafts had not been irradiated the percentages of healthy and tumour-free animals were 23% (controls 0) and 74% (controls 5%) respectively.

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* The exposures were kindly carried out for us by Miss Gladwys Scott.

These results must not be taken to imply that no resistance is produced by intra-splenic immunisation with irradiated tumour, for all the controls that were subcutaneously inoculated with non-radiated tumour died of growth. It appears to indicate, however, that with intra-splenic immunisation a smaller degree of initial and no greater degree of terminal general resistance to a later subcutaneous test inoculation is conferred when that immunisation is made with heavily radiated than when it is made with non-radiated material.

In this connection it is noteworthy that for no readily assignable reason deaths of the animals soon after intra-splenic inoculation with irradiated material were, in our experience, markedly more numerous than when non-radiated material was used intra-splenically, or radiated or non-radiated material was inoculated subcutaneously or intra-cerebrally. In a total of 348 rats operated upon by the same one of us (R. H. P.), the deaths for no readily-assignable reason during the first seventeen days after the first inoculation were as follows: Subcutaneous inoculations—non-radiated material 2%, irradiated 3%; intra-cerebral inoculation—non-radiated material 6%, irradiated 8%; intra-splenic inoculation—non-radiated material 8%, irradiated 31%.

COMPARISON BETWEEN THE EFFECTS OF INTRA-SPLENIC, INTRA-CEREBRAL AND SUBCUTANEOUS INOCULATION.

In another place, we, Lazarus-Barlow and Parry (1923), have brought forward evidence to show (1) that an intra-cerebral graft meets with a degree of local resistance to its growth which does not obtain in the case of a subcutaneous graft, and (2) that a greater degree of general resistance to later subcutaneous inoculation follows intra-cerebral immunisation than follows one beneath the skin. While the experiments that form the subject of the present paper were being carried out, intra-cerebral inoculations were made on series of 10 rats for the purpose of comparison. These confirmed our previous conclusions, and in addition gave evidence of a greater degree of *local* resistance in the spleen than in the brain, and of a greater degree of *general* resistance to a later subcutaneous inoculation after an intra-splenic than after an intra-cerebral immunisation.

The evidence for a greater *local* resistance in spleen than in brain consists in the fact that whereas in Exp. 1* 100% and in Exp. 2, 83% of intra-cerebral grafts developed tumours, only 59% and 21% of intra-splenic grafts followed this course.

The contrast between local resistance in spleen and in subcutaneous tissue is even more striking, as is shown in Table V.

TABLE V.

Condition of graft.	"Takes" per cent. of cases. Intra-splenic.	 Subcutaneous.
Fresh	59	100
,,	21	90
,,	77(¹)	95
Irradiated	0	26(²)
,,	0	10(²)
Frozen and thawed	0	30(²)

(¹) Subcutaneous tumour present when inoculated.
(²) Four weeks' delay in appearance of growths.

The evidence for a greater degree of *general* resistance to a later subcutaneous inoculation, conferred by intra-splenic as contrasted with intra-cerebral immunisation, is afforded by the fact that in Exp. 1, none, and in Exp. 2, 10% of the rats that had received intra-cerebral grafts were healthy and tumour-free at the conclusion of the experiment, whereas of those which had received intra-splenic immunisation 23% and 74% respectively were healthy and tumour-free. When heavily irradiated material was used for the intra-cerebral or intra-splenic immunisation a similar contrast between the two organs was observed.

Using the same criterion and combining all the available experiments detailed in this paper, 22 out of 95 rats (23%) that had received subcutaneous immunisation were healthy and tumour-free at the end of the experiment, whereas for those which had received intra-splenic immunisation the numbers were 30 out of 68 (44%). Eliminating experiments in which irradiated tumour was used for immunisation, the value for subcutaneous immunisation was 4% and for intra-splenic immunisation 46%.

THE DEGREE OF GENERAL RESISTANCE, WHEN PRESENT, CONFERRED BY A PRELIMINARY INOCULATION.

In the preceding sections frequently it has been stated that animals were "healthy and tumour-free" at the end of the experiment. In many instances this fact could only be determined after killing the animal, but in others the deduction was made from the general condition of the rat. Rats of the latter category were subjected to a third inoculation (subcutaneous) with an actively growing tumour to determine the degree of immunity that was present. 51 of such animals were re-inoculated at periods varying from two weeks to two months after a second inoculation. In 48 of these the graft did not develop at all, or developed for a short time and subsequently disappeared. In the remaining 3 a small, slowly-growing tumour appeared beneath the skin, was present when the animal was killed, and presented normal histological features. The immunity induced is, therefore, of a high order.

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* The local resistance presented by brain was, in this experiment, manifested by delay in growth alone and not by failure of the graft to take in addition to delay. Inoculated subcutaneously the tumour mortality was 100%.

HISTOLOGY OF SPLEEN.

Routine histological examination was made of the spleen, and in 59 cases glycerine films of the pulp were prepared for cell examination. No very definite results accrued from this work. The most that can be said is that when fresh grafts had been inoculated into spleen the splenic cells were larger and there was a larger number of pyronin (plasma) cells under Pappenheim's stain than when irradiated material had been used. The occurrence of these pyronin-staining cells appears to be associated with the presence of actual tumour growth in the spleen, for there was found on an average 1 such cell to 12 other splenic cells when there was a complete absence of growth in the body, or when growth was present elsewhere in the body but absent from the spleen, whereas the ratio was 1 : 7 when the spleen itself was the seat of growth. In this connection it is noteworthy that the cytoplasm of Jensen's rat sarcoma cells has a marked avidity for pyronin. No correlation between the number of pyronin-staining cells and the degree of general immunity was detected and no unusual types of cell were found. Whether fresh or irradiated material was used as graft the number of typical lymphocytes in the spleen appeared to be unusually small. Though definite measurements are not available, the spleen was certainly much larger in cases of intra-splenic than in cases of intra-cerebral inoculation, even though the intra-splenic graft itself failed to develop. Indeed the organ appeared to be larger than normal with intra-splenic and smaller than normal with intra-cerebral inoculation.

CONCLUSIONS.

We conclude in the case of the rat and Jensen's rat sarcoma that :

(1) The spleen offers a greater local resistance to growth of a tumour graft than brain or subcutaneous tissue.

(2) After intra-splenic immunisation the general resistance to later subcutaneous inoculation is raised, and that to a greater extent than after intra-cerebral or subcutaneous immunisation.

(3) Intra-splenic inoculation retards growth in an established subcutaneous tumour but does not lead to its disappearance.

REFERENCES.

Chambers, H., Scott, G., and Russ, S.—(1922) *Lancet*, **1**, 212.
Haaland, M.—(1910) *Proc. R. Soc.*, B., **82**, 293.
Kettle, E. H.—(1912–13) *J. Path. & Bact.*, **4**, 40.
Lazarus-Barlow, W. S., and Parry, R. H.—(1923) *Brit. J. Exper. Pathol.*, **4**, 80.

SPONTANEOUS DESENSITISATION.*

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THE early experiments of Rosenau and Anderson (1909), who found that guinea-pigs two and even three years after the injection of horse-serum were still extremely sensitive to the intravenous injection of the antigen, led them to the view, which is still generally held, that anyphylactic sensitiveness produced by a single injection of alien protein persists throughout the life of the animal. In some experiments on immune guinea-pigs Dale (1913) noted that the plain muscle, three months after the last immunising injection, had largely lost its sensitiveness. More recently Thomsen (1917) has shown quantitatively that actively anaphylactic guinea-pigs undergo a definite diminution of sensitiveness with the lapse of time—a change which depends only in part upon the protection against the injection of antigen which is afforded them by their increasing age and weight. Weil (1913) demonstrated the difference which exists between guinea-pigs sensitised passively with homologous and those sensitised with heterologous immune serum. The former were still sensitive on the 70th day, while in the latter sensitiveness to the antigen disappeared in a few days, and was replaced by active sensitiveness towards the heterologous serum proteins.

Our experiments concern (1) the influence of partial immunisation produced by very small doses of antigen in actively sensitive guinea-pigs upon the rate at which spontaneous desensitisation occurs, and (2) the rate of spontaneous desensitisation in guinea-pigs passively sensitised with homologous immune serum.

SPONTANEOUS DESENSITISATION IN ACTIVELY SENSITIVE GUINEA-PIGS.

A large series of guinea-pigs were actively sensitised by the subcutaneous injection of 1 mg. of crystalline horse-serum albumin. Some of these were subjected to no further treatment, and the tests of their sensitiveness one month and twelve months after the sensitising injection gave us information as to the extent to which spontaneous desensitisation occurred in this series of actively sensitive animals, and at the same time served as a control for the further experiment concerning the influence of immunisation. One month after the sensitising dose tests by the intravenous injection of antigen gave the following results :

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* The experiments described in this paper were carried out while Kellaway was Foulerton Student of the Royal Society, and their cost was defrayed by a grant from the Graham Research Fund.

Wt. of G.P.	Dose of antigen.	Result.
265 gm.	0·1 mg.	Slight symptoms, recovery.
255 gm.	0·2 mg.	† in 3½ minutes.
330 gm.	0·2 mg.	† in 3 minutes.

M.L.D. = 0·2 mg.

One year after the sensitising injection the animals were much larger and their sensitiveness had greatly diminished, as the following results show:

Wt. of G.P.	Dose of antigen.	Result.
550 gm.	0·5 mg.	Moderate symptoms, recovery.
540 gm.	2·0 mg.	Severe symptoms, recovery.
500 gm.	5·0 mg.	,, ,, ,,
600 gm.	20·0 mg.	† in 5¼ minutes.

A second dose of 10 mg. of antigen ten minutes after the first injection failed to cause any symptoms in the first two animals in this table. The M.L.D. lies somewhere between 5 and 20 mg., and is probably about twenty times as great per kilo of body-weight as it was eleven months before. During this period the animals had doubled their weight, and the large size of their bronchial tubes doubtless helped to protect them from any but large doses of antigen.

In Thomsen's experiments, in which horse-serum was used as the antigen, a similar degree of spontaneous desensitisation occurred in the course of a year. By testing the sensitiveness of the plain muscle by Dale's (1913) method we were able to avoid the complicating effect of the increase in size of the animals, and to make our results quantitative without the use of a very large number of guinea-pigs. Two months after the sensitising injection the plain muscle gave a small reaction to a concentration of 1 in 1,200,000 of the antigen, but reacted maximally to 1 in 240,000. Ten months later a concentration of 1 in 12,000 of the antigen was required to produce a maximal response and only a small reaction occurred to 1 in 240,000.

Spontaneous desensitisation in guinea-pigs is, therefore, a very slow process. We have not followed the change beyond the first year after the acquirement of sensitiveness and are unable to say whether it is ever complete.

THE INFLUENCE OF PARTIAL IMMUNISATION ON SPONTANEOUS DESENSITISATION IN ACTIVE ANAPHYLAXIS.

A number of the anaphylactic guinea-pigs of this series one month after the sensitising dose were temporarily desensitised by the subcutaneous injection of 1 mg. of crystalline horse-serum albumin. They were then partially immunised by an intravenous injection of 0·5 mg., followed by a series of five subcutaneous injections of 1 mg. of horse-serum albumin at weekly intervals. One or more of these animals were killed at various intervals after the last immunising injection, and their plain muscle was perfused and tested. The results are given in the following table. It should be noted that the animals tested one month after immunisation received only two immunising injections.

Time in relation to immunisation.	Dose of antigen (horse-serum albumin).	Reaction of plain muscle.
Before	0·5 mg.	Maximal.
	0·1 mg.	Small.
1 month after	0·5 mg.	Maximal.
	0·1 mg.	Maximal.
	0·02 mg.	Small response.
2 months after	0·5 mg.	Maximal.
	0·1 mg.	Large, but poorly sustained response.
4 months after	1·0 mg.	Small, modified response.
	10·0 mg.	Large, but poorly sustained response.
6 months after	1·0 mg.	Very small response.
	10·0 mg.	Maximal.
8 months after	10·0 mg.	Moderate poorly sustained response.
	20·0 mg.	Maximal in extent, poorly sustained.
9 months after	10·0 mg.	*Nil.*
	20·0 mg.	*Nil.*
	30·0 mg.	Very small.

The extent of the anaphylactic response of the plain muscle was estimated in all cases by comparison with the contraction obtained by the subsequent addition of 0·05 mg. or 0·1 mg. of histamine to the bath, which contained 120 c.c. of oxygenated Ringer's fluid. The decrease in the sensitiveness of the plain muscle of these partially immunised guinea-pigs is more marked than that which occurs in untreated actively anaphylactic animals of the same series. Whereas one month after partial immunisation a maximal reaction was obtained to a concentration of 1 in 1,200,000, nine months after the last immunising injection the plain muscle gave only a minute response to a concentration of 1 in 4000 of the antigen. At this time, a year after sensitisation, the plain muscle of untreated actively sensitive guinea-pigs of this series reacted maximally to a concentration of 1 in 12,000 of the antigen, so that even the very partial immunisation conferred by the injection of small doses of antigen was effective in accelerating the normal slow rate of spontaneous desensitisation.

The extent to which these animals were immunised was shown by the capacity of their sera to confer passive sensitiveness. 2 c.c. of the serum from an animal two months after the last immunising injection, administered intra-peritoneally to a normal guinea-pig weighing 250 gm., produced in 48 hours such a degree of sensitiveness that the plain muscle reacted maximally to a concentration of 1 in 60,000 and almost maximally to 1 in 120,000 of horse-serum albumin. The plain muscle of a normal guinea-pig which had been injected 48 hours before with 5 c.c. of this serum reacted maximally to a concentration of 1 in 240,000, and gave a large response to 1 in 1,200,000 of the antigen. 5 c.c. of the serum from an animal four months after immunisation contained much less antibody, since the plain muscle of the passively sensitive guinea-pig injected with this dose of serum gave only a moderate response to a concentration of 1 in 40,000 of the antigen. At later periods after immunisation we were unable to demonstrate any antibody in the serum by passive sensitisation experiments.

In the course of these experiments we made some observations on the duration of specific desensitisation after a single subcutaneous injection of 1 mg. of antigen. Seven male guinea-pigs of the same series 30 days after the sensitising injection were specifically desensitised in this way. They were tested by the intravenous injection of antigen at various periods with the following results:

Weight of G.P.	Days after densensitisation.	Dose of antigen.	Results.
295 gm.	2	0·2 mg.	Slight symptoms; recovery.
270 gm.	5	0·2 mg.	*Nil.*
250 gm.	8	0·2 mg.	Moderate symptoms; recovery.
350 gm.	9	0·2 mg.	Slight symptoms; recovery.
325 gm.	9	0·5 mg.	Very severe symptoms; only just recovered.
280 gm.	12	0·2 mg.	Slight symptoms; recovery.
260 gm.	12	0·5 mg.	Very severe symptoms; recovery after 2 hours.

The temporary and partial protection afforded by the subcutaneous injection of small doses of antigen lasted longer than we had anticipated. After 12 days there was still some protection to 2½ M.L.D. of the antigen.

Our experiments suggest that the method of protecting man from possible anaphylactic shock by repeated *small* subcutaneous injections of the alien protein to which he is sensitive, in addition to affording temporary and partial protection by specific desensitisation, has the advantage of greatly increasing the rate at which spontaneous desensitisation normally occurs.

SPONTANEOUS DESENSITISATION IN GUINEA-PIGS PASSIVELY SENSITISED WITH HOMOLOGOUS SERUM.

Weil (1914) showed that antigen as well as antibody was present in the serum of guinea-pigs immunised against a foreign protein, and it seemed possible that persistence of sensitiveness in animals passively sensitised with such serum 70 days after the sensitising injection might be due to the development of active sensitiveness in response to traces of antigen in the serum. We attempted to discover if this were the case by testing, at various periods after sensitisation, the plain muscle of a series of guinea-pigs passively sensitised with immune guinea-pig serum. The immune serum used in these experiments was obtained by injecting a series of four intra-peritoneal and four subcutaneous doses of 0·5 c.c. of horse-serum into guinea-pigs at weekly intervals. Thirteen days after the last injection several of these animals were bled out and the pooled serum was titrated by testing its power to confer sensitiveness on normal guinea-pigs.

Wt. of G.P. in gms.	Dose of serum intraperitoneally.	Result of intravenous injection of 0·1 c.c. of horse-serum after 48 hours.
240	1·0 c.c.	† in 5½ minutes.
250	1·0 c.c.	† in 5¾ minutes.
240	0·5 c.c.	Very severe symptoms; just recovered.
240	0·5 c.c.	† in 4¼ minutes.
200	0·2 c.c.	*Nil.*

The minimal sensitising dose of this pooled serum (against a dose of 0·1 c.c. of horse-serum) was between 0·5 c.c. and 1·0 c.c.

A series of twelve female guinea-pigs were now sensitised by the intraperitoneal injection of 1 c.c. of this immune serum and their plain muscle was perfused and tested at various periods after injection, with the following results:

Time after injection of immune serum.	Dose of antigen (horse-serum).	Reaction of the plain muscle.
1 day	0·02 c.c.	½ maximal.
	0·1 c.c.	¾ maximal, poorly sustained.
2 days	0·01 c.c.	½ maximal.
	0·1 c.c.	¾ maximal.
4 days	0·01 c.c.	Nearly maximal.
	0·1 c.c.	Maximal.
6 days	0·004 c.c.	¾ maximal.
	0·01 c.c.	Nearly maximal.
9 days	0·01 c.c.	*Nil.*
	0·1 c.c.	Small response.
9 days	0·1 c.c.	¾ maximal.
	0·5 c.c.	Maximal.
11 days	0·01 c.c.	¾ maximal.
	0·5 c.c.	Maximal.
14 days	0·01 c.c.	*Nil.*
	0·5 c.c.	Nearly maximal.
16 days	0·5 c.c.	Maximal.
	0·01 c.c.	Very small.
23 days	0·01 c.c.	Very small response.
	0·5 c.c.	½ maximal.
31 days	0·01 c.c.	*Nil.*
	0·1 c.c.	*Nil.*
31 days	0·1 c.c.	*Nil.*
	1·0 c.c.	*Nil.*

The maximal sensitiveness which we detected was at the 4th and 6th days, when a concentration of 1 in 30,000 of horse-serum gave a $\frac{3}{4}$ maximal response, while the reaction to 1 in 12,000 was almost maximal. After this time there was a fall in sensitiveness, so that on the 14th day similar responses were only obtained to a concentration of 1 in 240 of horse-serum. By the 31st day the plain muscle was not sensitive to a concentration of 1 in 120 of the antigen. Stronger concentrations could not be tested, since in these horse-serum has a stimulant effect on plain muscle apart from its antigenic property. The reaction of the whole animal to the intravenous injection of horse-serum gave confirmatory results. Two days after the sensitising injection 0·1 c.c. of horse-serum invariably killed animals sensitised with 1·0 c.c. of this immune serum, whereas in 4 guinea-pigs tested 31 days after sensitisation the injection of 5·0 c.c. of horse-serum produced no anaphylactic symptoms.

Diminution of sensitiveness was not, within the time of our experiment, followed by an increase of sensitiveness, as might have been the case if passive were replaced by active sensitiveness. Spontaneous desensitisation occurred at a much more rapid rate than in actively anaphylactic animals.

There seems to be a definite tendency to spontaneous desensitisation in all forms of anaphylactic sensitiveness in the guinea-pig. The rate of desensitisation is most rapid in passive sensitiveness with heterologous serum, occurring in a few days (Weil). In animals passively sensitised with homologous serum, sensitiveness in our experiments was not demonstrable after 30 days. In actively anaphylactic guinea-pigs there is a marked decline in sensitiveness during the first year after sensitisation, and this slow spontaneous desensitisation is greatly accelerated by immunisation.

REFERENCES.

Dale, H. H.—(1913) *J. Pharm. and Exp. Therap.*, **4**, 195.
Rosenau, M. J., and Anderson, J. F.—(1909) *Hyg. Lab. Bull.*, No. 50.
Thomsen, O.—(1917) *Ztsch. f. Immunitätsforsch.* (Orig.), **26**, 213.
Weil, R.—(1913) *J. Med. Res.*, **27**, 509 ; **28**, 259.—(1914) *Ibid.*, **30**, 363.

THE ACID-BASE BALANCE IN THE PLASMA IN THE LATER STAGES OF PREGNANCY.

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HASSELBALCH and Gammeltoft (1915) found evidence that the blood was less alkaline during the later stages of pregnancy than after delivery. Losee and Van Slyke (1917) also found that the plasma bicarbonate during pregnancy was below normal. These results indicate that there is in pregnancy an accumulation of acid in the blood or depletion of alkali, although Losee and Van Slyke's results are inconclusive, as they did not allow for the possibility of reduction of plasma bicarbonate produced by reduction of CO_2 tension by hyperpnœa.

Low values for plasma bicarbonate may be due to accumulation of acid in or loss of base from the blood, causing a relative excess of acid which combines with some of the base usually present as bicarbonate; or due to migration of Cl-ions out of the red blood-corpuscles in response to reduction of the CO_2 tension of the blood, in which case the base of the usual bicarbonate is used up to combine with these Cl-ions, although there is no excess of acid in the blood as a whole.

These two conditions may be distinguished by determining also the CO_2 tension or the hydrogen-ion concentration of the plasma. It is convenient to use a diagram as in Fig. 1; in this ordinates represent concentration of bicarbonate in plasma in gramme equivalents per litre, abscissæ CO_2 tension in mm. of mercury. A point representing a plasma is fixed if the plasma CO_2 tension (abscissa) and bicarbonate (ordinate) are known. Since the H-ion concentration is proportional to the ratio CO_2 tension : plasma-bicarbonate, points representing plasmata with the same H-ion concentration lie on straight lines passing through the origin; such lines are shown in the diagram, calculated according to the formula given by Austin *et al.* (1922), and designated in terms of pH. The point representing a plasma can also therefore be fixed if the bicarbonate (giving the ordinate) and pH (giving the diagonal line on which the point lies) are known.

If samples of plasma are separated from the same blood at varying CO_2 tensions, points representing these samples of plasma will lie on a curve such as A_1, A_2 (which may be called a true plasma dissociation curve). If from the same person samples of blood are taken (1) after normal breathing when the CO_2 tension is about 50 mm., and (2) after excessive lung ventilation when the venous CO_2 tension is 30 mm., the bicarbonate content of plasma separated from these two samples of blood will be 0·0275 and 0·0225 gm. equivalents per litre. But if we know the pH of these plasma samples we can fix the points representing them, and, finding that they lie on the same curve, conclude that the reduction of plasma bicarbonate is due only to reduction of

CO_2 tension. If, on the other hand, non-volatile acid accumulates in the person's blood, the plasma bicarbonate at a given CO_2 tension will be lower, and the dissociation curve will be lower than before; this is illustrated by the points K_1 and K_2, representing the plasmata of two patients with moderate and severe ketosis (0·094 and 0·305 gm. of acetone bodies as β-hydroxybutyric acid per litre).

Methods.—The subjects of this investigation were pregnant women attending the antenatal clinic of the London Hospital; no pathological changes had been found on clinical examination. The blood was taken between 11.45 a.m. and 12.15 p.m. and the plasma separated without loss of CO_2 as described by one of us (Marrack, 1923). The plasma so separated was used for all the estimations.

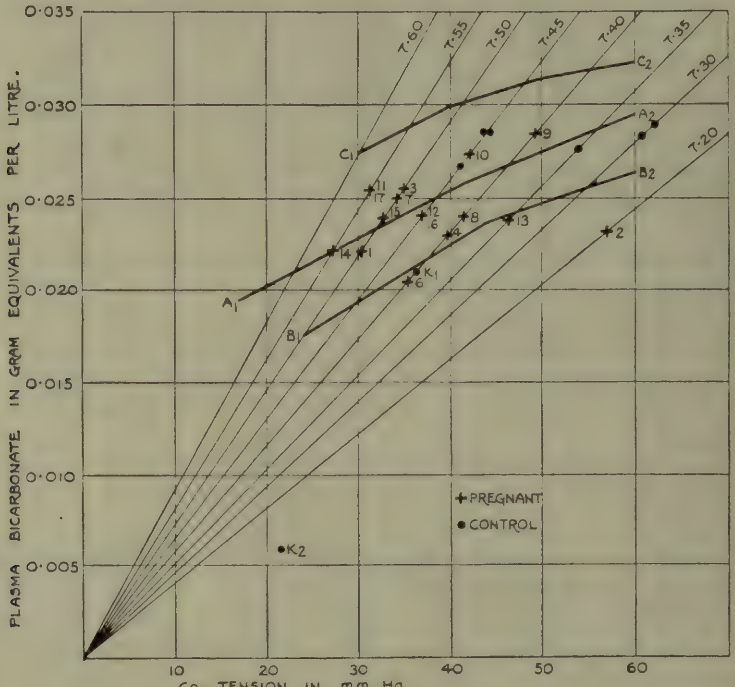

FIG. I.

Hydrogen-ion concentration and plasma bicarbonate were determined by the methods of Cullen (1922) and Van Slyke (1922): chlorides, by boiling with excess of silver nitrate in the presence of nitric acid and titrating the excess of silver nitrate with potassium thiocyanate; sodium, by the direct method of Kramer and Tisdall (1921), checked by the incineration method of Kramer (1920). It was found essential to separate the plasma at once from the corpuscles, to avoid low values owing to diffusion of sodium into the corpuscles.

As controls six young women students and graduates of this hospital were bled shortly before lunch, at times from 11.30 a.m. to 12.30 p.m. It must be remembered in interpreting the results that these controls were of exceptionally good physique and therefore not strictly comparable with the hospital outpatients, although these also were above the standard of ordinary hospital patients.

The true plasma dissociation curves given by normal bloods lie between B_1, B_2 and C_1, C_2; this range is calculated from the whole blood dissociation curves given by Peters, Barr and Rule (1921). The six controls come well within these boundaries, and lie very close to each other so far as height of curve is concerned. As may be expected in venous blood, the CO_2 tension and pH vary.

TABLE I.

Nº	NUMBER OF WEEKS PREGNANT	pH	HCo_3^-	Cl^-	$HCo_3^- + Cl^-$	Na^+	ACETONE BODIES	URINE
			GRAM EQUIVALENTS PER LITRE					
1	36	7·50	0·022	0·106	0·128	0·135	0·001	
2	36	7·25	0·023	0·1025	0·1255	0·133	0·0015	HAZE OF ALBUMEN. ACETONE.
3	37	7·50	0·0255	0·1045	0·130	0·1305	0·001	TRACE OF ALBUMEN. ACETONE
4	38	7·40	0·023	0·106	0·129	0·1305		
5	38	–	0·023	0·100	0·123	0·1305		TRACE OF ALBUMEN.
6	32	7·40	0·0205	–	–	–		
7	32	7·50	0·025	0·100	0·125	0·1305	0·001	
8	34	7·40	0·024	0·101	0·125	0·1305	0·001	ACETONE.
9	32	7·40	0·0285	0·095	0·1235	0·135		
10	30	7·45	0·0275	–	–	0·128		HAZE OF ALBUMEN. FEW GRANULAR CASTS.
11	35	7·55	0·0255	0·106	0·1315	–		
12	36	7·45	0·024	0·1015	0·1255	–		
13	32	7·35	0·024	0·0975	0·1215	0·1305		GLUCOSE PRESENT. BLOOD SUGAR 0·10 PER CENT.
14	22	7·55	0·022	0·101	0·123	0·1305		
15	38	7·50	0·024	0·1005	0·1245	0·1305		
16	36	7·45	0·024	0·103	0·127	0·135		HAZE OF ALBUMEN. FEW GRANULAR CASTS.
17	32	7·55	0·0255	0·100	0·1255	0·129		
AVERAGE OF CASES IN WHICH HCo_3^- Cl^- AND Na^+ WERE DETERMINED			0·0240	0·1009	0·1249	0·1321		

The points representing the plasmata of eleven of the sixteen pregnant women lie on curves of the same level as the controls*; of the other five, only two lie below the normal boundaries. But the pH was distinctly higher and the CO_2 tension lower than in the controls (average CO_2 tension of controls 52·1 mm., of pregnant women 38·3 mm.). This agrees with the low alveolar air CO_2 tensions found by Rowe (1923) in pregnant women. The values found for plasma bicarbonate are rather low, 0·024 gm. equivalents per litre instead of 0·0277, or, using the old method of expression, 54 c.c. of CO_2 per 100 c.c. of plasma instead 62 c.c. This is as it should be in plasmata derived from blood at low CO_2 tensions.

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* The inference of the height of the dissociation curve from one point assumes that the curves of the cases under consideration do not differ notably in slope from normal; appreciable deviation from normal is only likely to occur in severe anæmia, which was not present in any of our patients. The curves found by Hasselbalch and Gammeltoft (1915) in pregnancy, determined by two points, were of normal slope.

Acetone bodies were present in the urines of some of the subjects, but in those cases in which the acetone bodies in the blood were estimated, the excess found above normal (about 0·0005 gm. equivalents per litre) was not enough to cause appreciable reduction of plasma bicarbonate.

In Tables I and II are given the concentrations of HCO_3, Cl and Na-ions in these plasmata. From these data the balance of anions and kations in the plasmata of pregnant women and controls may be compared. In pregnant women as in other people the concentrations of HPO_4, K, Ca, and Mg-ions (Krebs and Briggs, 1923) vary little from 0·0016, 0·005, 0·005 and 0·0025 gm. equivalent per litre. The sum of all the kations in the plasma can be obtained from the figures for Na by adding 0·0125. This gives in the controls an excess of kations unaccounted for by HCO_3, Cl, and HPO_4-ions, equivalent to 0·019: most of this is combined with protein; in the pregnant women the excess is 0·018. These figures probably agree within the limits of error in Na-ion estimations, but Dr. Coetzee found in these cases that the plasma protein of the pregnant women was less than that of the controls (7·39 instead of 8·05 per cent.); so that a difference of the order found in the amounts of kation combined with protein, and therefore of the amounts of kation unaccounted for by HCO_3, Cl, and HOP_4-ions, is to be expected. If appreciable amounts of abnormal acid were present in the plasmata of pregnant women this should reveal itself by an abnormal excess of kations; this we have not found.

TABLE II.

pH	HCO_3^-	Cl^-	$HCO_3^- + Cl^-$	Na^+
	GRAM EQUIVALENTS PER LITRE			
7·35	0·0275	0·100	0·1275	0·135
7·45	0·0265	0·104	0·1305	0·139
7·45	0·0285	0·100	0·1285	0·137
7·45	0·0285	0·1006	0·129	0·141
7·30	0·0290	0·098	0·127	0·135
7·30	0·0285	0·104	0·1325	0·141
AVERAGE	0·0277	0·102	0·1297	0·138

Our results differ most from those of Hasselbalch and Gammeltoft in that we found as a rule an alkalæmia; this difference is reflected in the urines of the patients; those which we collected corresponding to the period in which the blood was taken were neutral or alkaline, while those of Hasselbalch and Gammeltoft's patients were strongly acid. The lowering of the dissociation curve which they found is not great, as in only three out of eight pregnant cases is the pH at 40 mm. CO_2 more than 0·01 (a difference we should not detect) below that of the blood of Hasselbalch himself.

Such lowering of the dissociation curve as we have found appears to be due to deficiency of alkali rather than accumulation of abnormal acids in the blood.

CONCLUSION.

In the latter half of pregnancy there is as a rule a reduction of CO_2 tension in the blood; this produces a moderate alkalæmia and reduction of plasma bicarbonate. In a minority of cases there is an actual alkali deficit.

We owe our thanks to the physicians of the Ante-Natal Clinic of the London Hospital for the facilities they have afforded for this investigation.

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REFERENCES.

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D.—(1922) *J. Biol. Chem.*, **54**, 144.

Cullen, G. E.—(1922) *ibid.*, **52**, 501.

Hasselbalch, K. A., and Gammeltoft, S. A.—(1915) *Biochem. Ztsch.*, **68**, 206.

Kramer, B.—(1920) *J. Biol. Chem.*, **41**, 263.

Kramer, B., and Tisdall, F. F.—(1921) *ibid.*, **46**, 467.

Krebs, O. S., and Briggs, A. P.—(1923) *Am. J. Obst. & Gyn.* **5**, 67.

Losee, J. R., and Van Slyke, D. D.—(1917) *Am. J. Med. Sci.*, **153**, 94.

Marrack, J.—(1923) *Biochem. J.*, **17**, 240.

Peters, J. P., Barr, D. P., and Rule, F. D.—(1921) *J. Biol. Chem.*, **45**, 571.

Rowe, A. W.—(1923) *ibid.*, **55**, 347.

Van Slyke, D. D.—(1922) *ibid.*, **53**, 495.

THE CLASSIFICATION OF HÆMOGLOBINOPHILIC BACTERIA, BASED UPON THEIR RELATION TO BLOOD-PIGMENT AND TO THE "VITAMINE" FACTOR.*

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In their attempt to classify micro-organisms more closely upon a botanical-biological basis the Society of American Bacteriologists (1920) have coined a new genus, "Hemophilus," which provisionally includes such diverse bacteria as *B. influenzæ*, *B. pertussis*, *B. duplex*, Ducrey's bacillus and the Koch-Weeks' bacillus. These bacteria are described as "minute rod-shaped cells . . . growing best (or only) in the presence of hæmoglobin, and in general requiring blood-serum or ascitic fluid."

In this genus, therefore, are included not only the "hæmoglobinophilic" bacteria, but also others which merely have a preference for *blood* in the medium or even for serum or for ascitic fluid.

The biological aspect of this classification does not take into account the leading character of a hæmoglobinophilic bacterium, typified by *B. influenzæ*, namely its dependence for growth upon an iron-containing blood-pigment as pointed out by Pfeiffer in his original paper. As is well known, neither *B. pertussis* nor *B. duplex* are dependent upon blood-pigment.

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* Work carried out under a grant from the Medical Research Council.

In more recent years the conditions of growth of *B. influenzæ* have been more closely established. It has been confirmed that blood-pigment is necessary (or according to some, an unspecified heat-stable factor in blood), but it is also found that a heat-labile factor, usually known as the "V" factor, is equally necessary (Davis, 1921; Fildes, 1921; Rivers and Poole, 1921; Thjötta and Avery, 1921[1]).

These characters of *B. influenzæ* are so sharply defined that it appears undesirable to include in the same group as this bacillus other bacteria which have no such dependence upon special food factors, but merely a preference for blood or ascitic fluid when first isolated from the body.

THE RELATION TO GROWTH FACTORS OF CERTAIN SO-CALLED "HEMOPHILIC" BACTERIA.

The degree of dependence upon hæmatin and V factor exhibited by some of the "hemophilic" bacteria and by *B. hæmoglobinophilus canis*, not referred to by the American Committee, was determined as follows:

Eight strains of *B. influenzæ*, obtained from the lungs of cases of pneumonia *post mortem*, were compared with 23 strains of Koch-Weeks' bacillus from patients with muco-purulent conjunctivitis; with 5 recently isolated strains of *B. duplex*; with 4 strains of *B. pertussis* obtained from the National Collection of Type Cultures, and with one strain of *B. hæmoglobinophilus canis* sent to me by Dr. Martin Kristensen, of Copenhagen (Nat. Coll. Type Cultures, No. 1659).

Experiment 1.—The relation of various bacteria to growth factors.

For testing each strain 4 tubes of 1½ per cent. bacto-peptone water were taken. These contained no V factor. To two of the four tubes 0·1 c.c. of a 1 per cent. solution of hæmatin in water was added. This hæmatin was prepared from pure crystallised horse hæmoglobin (for which I have to thank Prof. H. E. Roaf) by drying with anhydrous potassium carbonate, extraction with alcohol and solution of the extract in water. To one of the tubes containing hæmatin and to another peptone water tube 1 c.c. of yeast extract was added. This yeast extract was made according to the directions of Thjötta and Avery (1921[1]), but filtered through a Berkefeld N candle to remove traces of X factor which are adsorbed to suspended particles of yeast.

These 4 tubes were then inoculated with one drop of a very fine suspension of the strain to be tested. The success of all experiments upon growth requirements of hæmoglobinophilic bacteria depends upon absence of hæmatin from the inoculum. The inoculation must obviously be made from a medium containing blood-pigment, but (*a*) the amount of blood in the medium should be as small as possible, viz. 1 per cent. of Fildes' (1920) peptic digest in agar, and (*b*) the culture should be young to reduce absorption of hæmatin upon the bacteria to a minimum, while (*c*) the quantity transferred should be minute, viz. the point of a needle (not loop) is touched upon a slope culture and agitated in 10 c.c. of salt solution; of this one drop is inoculated into each tube of peptone water.

The growth was allowed to continue for two days, or four days in the case of *B. pertussis*, and then subcultures of one loopful were transferred to Fildes' peptic blood agar, or to ordinary blood agar for *B. pertussis*, to determine the nature of the growth or the absence of growth.

The results with all strains of *B. influenzæ* and Koch-Weeks' bacillus were the same, while those with *B. hæmoglobinophilus canis* and the other bacteria were different. Two of the strains of *B. pertussis* would not grow under these conditions and were excluded.

	B. influenzæ.	Koch-Weeks.	*B. hæmoglobinophilus canis.*	*B. pertussis*, No. 760.	*B. pertussis*, No. 364.	*B. duplex* (3 strains).	*B. duplex* (2 strains).
Peptone-water + 0	0	0	0	+	+	+ +	+ +
,, + hæmatin	0	0	+ +	+	+ +	+ +	0
,, + yeast	0	0	0	0	+ +	+ +	+
,, + hæmatin + yeast	+ +	+ +	+ +	0	+ +	+ +	+ +

+ + = Visible growth in the peptone-water tubes and on subculture. + = No visible growth, but growth on subculture. 0 = No growth.

From this experiment it is clear that Koch-Weeks' bacillus has identical growth requirements to *B. influenzæ*, and may thus be associated with it in a classification based upon this character, while *B. hæmoglobinophilus canis* demands hæmatin but is indifferent to the V factor. It may be remarked that none of these strains were hæmolytic, while two of the influenza strains and one of the Koch-Weeks strains produced indol.

B. pertussis and *B. duplex* were in no way dependent for growth upon the factors which are essential for the influenza bacillus.

These results confirm those of Rivers (1922[1]). He tested the growth requirements of *B. influenzæ*, *B. pertussis* and *B. hæmoglobinophilus canis*, using peptone agar as the basal medium.

The demonstrated dependence of the hæmoglobinophilic organisms upon the iron-containing pigment hæmatin does not imply that other substances are not capable of exerting the same effect. Thjötta and Avery (1921[2]) have proved that potato can replace the hæmatin, which, according to Fildes (1922), is due to the fact that the actions of hæmatin and potato are similar. Again, it is known that a trivial growth of *B. influenzæ* may be obtained in impure culture with certain bacteria in the absence of hæmatin (Williams and Povitzky, 1921). This effect is also no doubt due to the peroxidase demonstrable in such bacterial cells by means of guaiaconic acid.

THE SIGNIFICANCE OF THE V FACTOR IN THE GROWTH OF THE HÆMOGLOBINOPHILIC BACTERIA.

The question whether the lack of dependence of *B. hæmoglobinophilus canis* upon the V factor is sufficient to exclude it from classification with *B. influenzæ* is difficult to decide in the absence of exact knowledge as to the identity of the V factor.

The V factor is not confined to the red blood-corpuscles, but is present in extracts from cells of the whole animal and vegetable kingdoms. It is probable, as has been suggested by Davis (1921) and Rivers and Poole (1921), that it is the V factor which is responsible for the well-known "symbiosis phenomenon" in the growth of the influenza bacillus. This phenomenon is not observed when excess of V factor is present in the medium.

The following experiments were carried out to throw light on the origin of V factor:

Experiment 2.—The relation of the symbiosis phenomenon to hæmatin and V factor.

The basal medium used was agar made with bacto-peptone, but containing no meat and not cleared with egg. Hæmatin and yeast were added as before. The sloped tubes were inoculated diffusely with a strain of *B. influenzæ* and then along one extreme edge with *Staphylococcus aureus*.

	Growth of *B. influenzæ*.		Growth of *B. influenzæ*.
Agar + 0	0	+ staphylococcus	0
,, + hæmatin	0	,,	symbiosis
,, + yeast	0	,,	0
,, + hæmatin + yeast	+ +	,,	diffuse

This experiment suggests that the symbiotic growth is due to a production of V by the staphylococcus in the presence of hæmatin. This type of growth is not apparent when V is already present in the medium.

Experiment 3.—The production of V factor in a medium from growth of *Staphylococcus aureus*.

A flask of bacto-peptone water, inoculated with staphylococcus and incubated for 48 hours, was filtered through a Berkefeld N candle and tested as follows:

Peptone-water—	Growth of *B. influenzæ*.	Staphylococcus filtrate—	Growth of *B. influenzæ*.
+ 0	0	+ 0	0
+ hæmatin	0	+ hæmatin	+ +
+ yeast	0	+ yeast	0
+ hæmatin + yeast	+ +	+ hæmatin + yeast	+ +

The filtrate of the culture thus contains the V factor which has been derived from the growth of the coccus.

Experiment 4.—The relation of the production of V factor to the life of the producing organism.

As is well known, V factor is often obtained by boiling yeast with water, but the proportion of yeast to water in this case is very large, and it is possible that the V extracted is obtained not so much from the yeast cells as from the extracellular products present in the commercial sample. A quantity of yeast was, therefore, washed by suspension in salt solution, collected upon a filter and thrown into acetone. The precipitated mass of yeast so obtained was dried and then extracted with water in the usual proportion. Although no exact estimation of the yield of V was made, nevertheless this factor appeared to be present in the usual quantity, and so must be supposed to have been extracted from the dead organisms.

On the other hand, experiments with staphylococcus indicate that this organism when present in small quantity has no stimulating action on the growth of *B. influenzæ* except when alive:

A mass of *Staphylococcus aureus* was centrifuged and thrown into acetone. The acetone was decanted, the deposit rinsed in ether and heated in dry air to 100° C. for 1 hour. This dry powder was rubbed up in agar in quantities sufficient to give a slight opacity. Hæmatin and yeast were added as before.

	Growth of *B. influenzæ.*		Growth of *B. influenzæ.*
Agar + 0	0	. + dead staphylococci .	0
,, + hæmatin . . .	0	. + ,, .	0*
,, + yeast . . .	0	. + ,, .	0
,, + hæmatin + yeast .	+ +	. + ,, .	+ +

* Usually after 24 hours a colony of staphylococcus develops, having survived the acetone and heating, and is surrounded by a well-marked symbiotic growth of *B. influenzæ*.

The living staphylococcus is thus the only source of V factor.

The same result is obtained as follows:

	Growth in subcultures.
Peptone-water + hæmatin + 0	0
,, ,, + staphylococcus, living . .	+ +
,, ,, + do., boiled 2 minutes . .	0
,, ,, + staphylococcus from acetone .	0
,, ,, + do., boiled 2 minutes . .	0
,, ,, + yeast	+ +

The growth of *B. influenzæ* was determined by subculture owing to the opacity of the tubes from the added cocci.

The conclusion may be drawn from these experiments that the production of V factor by the staphylococcus is almost entirely a function of the living organism. No doubt, if large quantities of cocci were boiled with a small quantity of water, as when a yeast extract is made, then V would also be demonstrable in the extract from the dead bodies of the cocci. In the presence of blood-pigment, the symbiosis effect is produced by the diffusion of V factor from a foreign colony in which it has been synthesised by the living organism.

This production of V factor would seem to be within the power of practically all bacteria to a greater or less extent. A large number of varieties have been tested by Kristensen (1922), with results which indicate that different species produce the same V factor, though in different amounts. On the other hand, *B. influenzæ* is an almost unique exception to this rule. As first pointed out by Davis (1921[3]), this organism has no power of stimulating the growth of influenza bacilli to produce a "symbiosis phenomenon," and so must be devoid of the power of synthesising V factor. This phenomenon was utilised by Davis as a test for influenza bacilli. With the present cultures it was found that neither *B. influenzæ* nor the Koch-Weeks bacillus had any stimulating action upon *B. influenzæ*, while the strain of *B. hæmoglobinophilus canis* had a marked effect (*cf.* Kristensen, 1922). This result is to be expected from the demonstrated growth requirements of these bacteria. Since *B. influenzæ* demands the addition of V factor, it is not to be expected that it would synthesise it. On the other hand, *B. hæmoglobinophilus canis* does not require V factor to be added, probably because it supplies it itself.

It may be suggested that it is this failure to synthesise V factor which is the outstanding feature of *B. influenzæ* separating it from other bacteria, rather than a requirement for V factor in growth.

CRITERIA FOR INCLUSION IN THE HÆMOGLOBINOPHILUS GROUP.

From the preceding discussion it follows that the leading characters of *B. influenzæ* are—first, an absolute dependence upon an iron-containing blood-pigment for growth, and secondly, a lack of ability to synthesise V factor. Both of these characters are also exhibited by the Koch-Weeks bacillus, while the absolute dependence upon blood-pigment is shared by *B. hæmoglobinophilus canis*, without, however, the inability to synthesise V factor.

The absolute dependence of these three organisms upon blood-pigment or upon animal or vegetable cell products which have a similar (catalytic) action seems to be sufficiently unique to allow of their inclusion in one group, but until the nature of this growth requirement is more definitely known it would appear undesirable to coin a new name to replace that of "hæmoglobinophilic." This group must clearly be sub-divided into two parts, the first containing *B. influenzæ* and the probably identical Koch-Weeks bacillus, both of which are incapable of synthesising the V factor, and the second embracing *B. hæmoglobinophilus canis*, which does not suffer from this disability. The other organisms included by the Society of American Bacteriologists in the "genus Hemophilus" should not be classified with the hæmoglobinophilic bacteria.

I have had no opportunity to examine strains of "hæmolytic hæmoglobinophilic" bacteria, but according to Kristensen (1922) these organisms are not always absolutely dependent upon blood-pigment for growth. On the other hand, Rivers (1922), using methods similar to those recorded here, has found that the growth requirements of the hæmolytic bacilli are the same as those of *B. influenzæ*.

CONCLUSIONS.

B. influenzæ, Koch-Weeks bacillus and *B. hæmoglobinophilus canis*, being absolutely dependent for growth upon blood-pigment or some substance with a similar action, may be classified together as a special "hæmoglobinophilic" group. This group is capable of sub-division into two sub-groups, the first containing *B. influenzæ* and the Koch-Weeks bacillus, and the second *B. hæmoglobinophilus canis*. The former are incapable of synthesising the V factor, while the latter is capable of so doing. Other bacteria described as "hemophilic" should not be classified with the hæmoglobinophilic group.

I have to thank Mr. C. B. Goulden, Ophthalmic Surgeon to the Hospital, for selecting the cases of conjunctivitis for me.

REFERENCES.

Davis, D. J.—(1921[1]) *J. Infect. Dis.*, **29**, 171.—(1921[2]) *Ibid.*, **29**, 178.—(1921[3]) *Ibid.*, **29**, 187.

Fildes, P.—(1920) *Brit. J. Exper. Pathol.*, **1**, 129.—(1921) *Ibid.*, **2**, 16.—(1922) *Ibid.*, **3**, 210.

Kristensen, M.—(1922) 'Investigations into the Occurrence and Classification of the Hæmoglobinophilic Bacteria,' Copenhagen (Levin & Munksgaard).

Rivers, T. M.—(1922[1]) *Bull. Johns Hopkins Hosp.*, **33**, 149.—(1922[2]) *J. Bacteriol.*, **7**, 579.

Rivers, T. M., and Poole, A. K.—(1921) *Bull. Johns Hopkins Hosp.*, **32**, 202.

Society of American Bacteriologists.—(1920) *J. Bacteriol.*, **5**, 191.

Thjötta, T., and Avery, O. T.—(1921[1]).—*J. Exper. Med.*, **34**, 97.—(1921[2]) *Ibid.*, **34**, 455.

Williams, A. W., and Povitzky, O. R.—(1921) *J. Med. Res.*, **42**, 405.

THE EFFECT OF LIGHT ON THE ORGANISM.

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The striking results of heliotherapy, especially in surgical tuberculosis and in rickets, contrast with our ignorance of the physiological action of light on the organism. Heliotherapy is as yet purely empirical and based on clinical observations. In this connection it may not be out of place to pay tribute to the pioneer work of Dr. O. Bernhard, of St. Moritz (Switzerland). His observations, which date from 1902, on the remarkable therapeutic effect of sunlight on the healing of wounds, generally led to the application of heliotherapy to cases of surgical tuberculosis. His observations are given in detail in his book 'Sonnenlichtbehandlung in der Chirurgie,' 1917, where an interesting and detailed account is given of the history of heliotherapy and of the general biological effects of light. He also states clearly there in 1917 that light has an important effect on the formation of bone, and attributes to its absence an essential part in the ætiology of rickets. He gives as an instance the seasonal distribution of rickets in a little mountain village (Bivio) on the Julier Pass, 1776 metres above sea-level, where the winter lasts nearly nine months. He found there that the children born in autumn, who had to be kept indoors in the dark houses with their narrow windows during the nine months of winter, nearly all developed rickets in varying degree, while children born in spring, whom the parents carried with them into the fields during the summer, escaped.

In the following attempt to investigate some aspects of the physiological action of light on the organism, we were guided by the known effects of the more powerful radiations from X-rays and radium, and the curious similarity which exists between these effects and those of vitamin deficiencies. It is known that radiations from X-rays and radium in moderate doses produce a lymphopenia, while very small doses produce a lymphocytosis. It is also one of the few observed physiological effects of light that it produces a lymphocytosis (Clark, 1922). We had found (Cramer, Drew and Mottram, 1922) that large doses of radiations from radium produced a decided fall in the number of platelets, and an even more decided thrombopenia had been observed by Fabricius-Möller (1922) as the result of massive doses of X-rays. As with lymphocytes, this atrophic effect could be replaced by a stimulating effect, by applying very small doses of radiations from radium; this produced a transitory increase in the number of platelets (Mottram, 1923[1]). Since, therefore, the platelets, like the lymphocytes, are susceptible to the influence of these radiations, it seemed possible that they might also be susceptible to the action of light. The point was tested by two different lines of investigation. Rats were kept in the dark since birth, and, after the effect of the absence of light on the platelets and other tissues had been determined, they were exposed to ultra-violet light. Since we had found previously that the number of platelets could be greatly diminished by a deficiency in the vitamin A, and increased by an abundant supply of this vitamin, the animals had to be kept on a diet which was constant with reference to its content in vitamin A, and which, moreover, contained a minimal supply of vitamin compatible with the absence of disease. In another set of experiments the effect of exposure to light was studied on rats, in which a great diminution in the number of platelets was produced by keeping them on a diet free from vitamin A. If light had a stimulating effect on the formation of blood-platelets, the exposure to light should tend to counteract the effect of the vitamin A deficiency. This set of observations was carried out in 1922 before it had been found that the onset of the general effects of vitamin A deficiency, such as arrest of growth and development of bacterial infections, could be delayed by exposure to light. A preliminary account of our results was given at a discussion on the physiological action of light (Cramer, 1922[1]).

EXPERIMENTAL.

Rats which have been kept in a dark room since birth do not show any obvious signs of ill-health. They grow approximately at the same rate as rats kept in a well-lighted animal room and fed on the same diet. Nevertheless they do differ from normal rats, and this difference manifests itself morphologically by an abnormally low number of blood-platelets. In our normal laboratory stock of rats kept on a diet of bread and water, boiled maize and rice the average number of blood-platelets in a normal rat lies roughly between 800,000 and 900,000 per cmm., while the number of red blood-corpuscles lies between 9 and 10 millions per cmm. The normal platelet : red cell ratio is therefore approximately 1 : 11.

The following table (Table I) gives the number of platelets and red cells in seven rats belonging to two litters, A and B, respectively. These two litters were born in a spacious room from which all daylight was excluded. Occasionally they received light for a few minutes from an ordinary electric bulb, which was switched on only whenever the room had to be lit for the feeding and weighing of the rats. They were kept in a deep wooden box with a lid of perforated zinc and fed on the ordinary laboratory diet mentioned above. The first counts were made when they were about three to four months old and had reached a weight of from 70 to 100 grm. The table shows that in six out of the seven rats examined the number of platelets was greatly subnormal. In one rat only (No. 489) the count gave normal figures. It is necessary to point out that the results were obtained with rats kept on a diet of known low vitamin content, namely, on our ordinary laboratory diet, which, though adequate to enable the rats to grow and to breed, is poor in vitamins, and produces a condition of "vitamin underfeeding" (Cramer, 1922[2]). On a vitamin-rich diet the stimulating effect of the fat-soluble vitamin counteracts the absence of light.

Table I

Nº of Rat and Colour	Exposure to Mercury vapour lamp	Length of Exposure	Number of Red Cells per c.m.m. in millions	Number of Platelets per c.m.m.	Platelet Red Cell RATIO	Date of Count	Weight in Grms
Litter A., born 6·6·22							
481	Before	—	8·1	340,000	1 : 23	18/9	75
White	After	14 days 3 each day	8·2	610,000	1 : 13	4/10	92
483	Before	—	7·1	370,000	1 : 19	24/10	67
White	After	25 days 5 each day	8·8	770,000	1 : 11	22/11	75
484	Before	—	6·6	280,000	1 : 24	4/10	70
Grey	After	18 days 3 each day	9·0	510,000	1 : 17	24/10	85
Litter B., born 4·6·22							
489	Before	—	8·9	890,000	1 : 10	4/10	100
White	—	—	—	—	—	—	—
490	Before	—	8·8	370,000	1 : 24	18/9	82
Wild • White	After	14 days 3 each day	8·6	660,000	1 : 13	4/10	107
491	Before	—	8·2	346,000	1 : 24	4/10	87
Wild	After	18 days 3 each day	9·1	552,000	1 : 17	24/10	95
493	Before	—	7·2	460,000	1 : 15	24/10	100
Wild	After	25 days 5 each day	9·0	710,000	1 : 13	22/11	120

After the first count had been made the rats with a subnormal number of platelets were exposed to the light of a mercury vapour lamp for 3 to 5 minutes each day for periods varying in different animals from 14 to 25 days. The arrangement for exposure to light was as follows: The rat was placed in a large wooden box, one side of which had been cut away and replaced by wide-meshed wire netting. The mercury vapour lamp was placed outside the wire netting and illuminated the whole box, in which the animal could move about freely. The counts were made in some cases on the same day as the last exposure but four hours later; in other cases two days after the last exposure, so that any temporary effects of exposure to light are excluded. The table shows in every case a very decided increase in the number of platelets, which was greatest in the two rats (Nos. 483 and 493) which had received the longest exposure to light. In these two rats the number of platelets after light treatment approximated the normal. In three rats (Nos. 483, 484, 493) a slight anæmia had developed before the light treatment. This anæmia disappeared after exposure to light.

It follows from these observations that *light has a stimulating effect on the formation of blood-platelets.* This conclusion was tested by studying the effect of light on the platelets of rats kept on a diet deficient in the fat-soluble vitamin A. We had found previously that on such a diet the number of platelets shows a progressive fall. If it be true that light had a stimulating effect on the formation of platelets, it should be possible to delay or counteract this thrombopenia of vitamin A deficiency by exposing rats kept on a diet deficient in vitamin A to ultra-violet light. Three such series of observations were carried out. In such experiments comparison can be made only if rats are used which have been bred in the laboratory and kept since birth on a known diet of a constant vitamin content. We have shown in several papers that rats kept on a vitamin-rich diet since birth resist a subsequent vitamin deficiency over a much longer period. This greater resistance manifests itself in the longer period which elapses before growth is arrested and before obvious pathological lesions develop (xerophthalmia, septic glands), and also in the slower fall in the number of platelets. For an experiment of this kind it is therefore essential to exclude as much as possible differences in resistance due to the preliminary dietary history of the animal.

Table 2

Vitamine A DEFICIENCY Total length of time in Weeks	Rat 530		Rat 531	
	Number in Thousands per c.m.m of		Number in Thousands per c.m.m. of	
	Red Cells	Platelets	Red Cells	Platelets
1	9.840	750	8.920	890
	↑ LIGHT ↓		↑ NO LIGHT ↓	
7	8.720	728	8.800	546
	↑ NO LIGHT ↓		↑ LIGHT ↓	
11	9.120	666	9.640	830

In our three series a comparison was made, therefore, in each series between rats not only of the same weight but also of the same litter which had been kept on a constant diet since birth.

Table 3

Vitamine A DEFICIENCY Total length of time in Weeks	Rat 442			Rat 443			Rat 444	
	LIGHT TREATMENT AFTER 7TH WEEK						NO LIGHT TREATMENT	
	Number in Thousands per c.m.m of			Number in Thousands per c.m.m of			Number in Thousands per c.m.m of	
	Red Cells		Platelets	Red Cells		Platelets	Red Gells	Platelets
7	9.880	↑	340	8,760	↑	415		
9	9.000		401	7.880		250	5.480	210
12	10.880	LIGHT	729	9800	LIGHT	780	7.480	240
15	9.160		575	7.520		380		
17	9.600	↓	657	8,840	↓	670		

The first two series of observations were made on rats from litters kept since birth on a vitamin rich diet. In one series (rats 530 and 531, Table II) one rat was exposed to light from a mercury vapour lamp almost from the beginning of the vitamin A deficiency for the first 7 weeks, the other being used as a control and receiving no light treatment. After this period the conditions were reversed. The results show that exposure to ultra-violet light, when applied early, delays the onset of thrombopenia; when applied later, after a thrombopenia has set in, the number of platelets increases again and may reach a normal level. It should be noted that in our previous observations on the effect of vitamin A deficiency

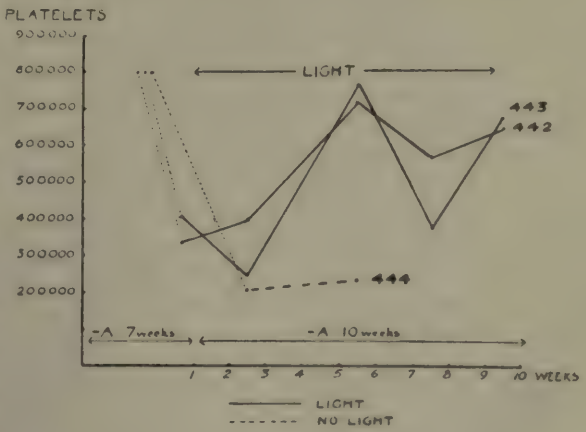

FIG. 1.

alone, the fall in the number of platelets was always progressive: after having reached a low level the number of platelets might become stationary, but never showed a decided increase.

In the second series (Nos. 442, 443, 444, Table III and Fig. 1) three rats from a litter kept on a vitamin-rich diet were placed on a vitamin A free diet. After 8 weeks, when all the rats had developed a deep thrombopenia, two of the rats (Nos. 442 and 443) received exposure to ultra-violet light, the third being kept as control and receiving no light treatment. In this third rat the deep thrombopenia was maintained, while in the other two the platelets showed a decided increase, so that even after 17 weeks of vitamin A deficiency the number approximated 700,000 per c.cm. One of these rats developed the typical eye lesion in the nineteenth week while still under the light treatment. In this experiment, which was actually our first attempt to study the effect of ultra-violet light, the mercury vapour lamp was used at first in its original armature, which emitted only a comparatively narrow cone of light from the centre of the quartz tube. After 8 weeks of light treatment the armature was removed so that the light from the whole length of the tube illuminated the cage in which the rats were exposed. It is possible that the fluctuations in the earlier part of the experiment are due to the imperfect lighting arrangement then used.

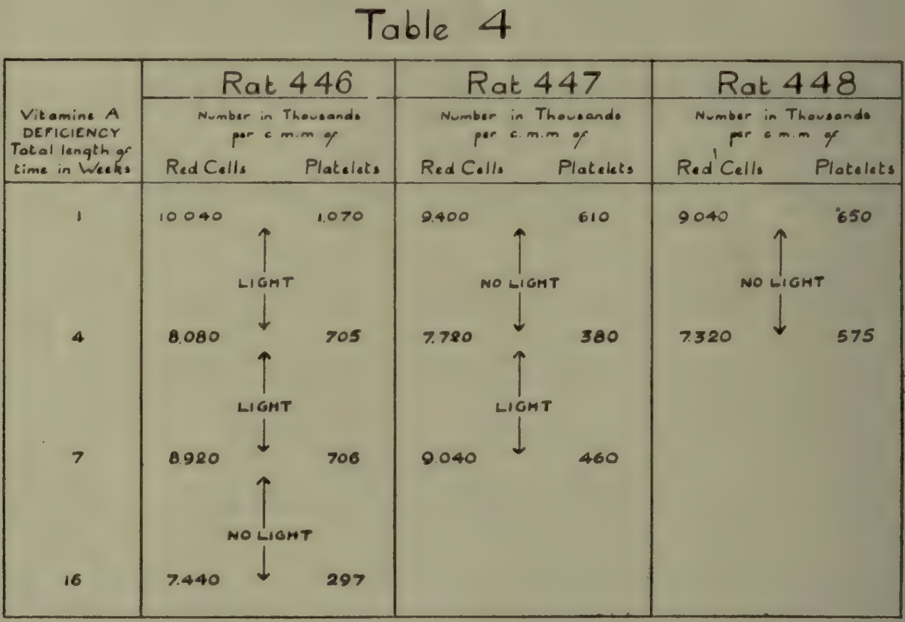
Table 4

Vitamine A DEFICIENCY Total length of time in Weeks	Rat 446		Rat 447		Rat 448	
	Number in Thousands per c.m.m of Red Cells	Platelets	Number in Thousands per c.m.m of Red Cells	Platelets	Number in Thousands per c.m.m of Red Cells	Platelets
1	10 040	1.070	9.400	610	9 040	650
	↑ LIGHT ↓		↑ NO LIGHT ↓		↑ NO LIGHT ↓	
4	8.080	705	7.720	380	7.320	575
	↑ LIGHT ↓		↑ LIGHT ↓			
7	8.920	706	9.040	460		
	↑ NO LIGHT ↓					
16	7.440	297				

The third series (Table IV) was carried out on rats from a litter which had been kept since birth on our ordinary laboratory diet. This diet, though containing vitamins, is so poor in these substances as to produce "vitamin underfeeding," which manifests itself in a slightly atrophic condition of the intestinal mucosa and hastens the development of the more profound atrophic lesion when vitamin A is completely withheld. The animals of this litter (Nos. 446, 447, 448) were so susceptible to vitamin A deficiency that of three rats which were put on the vitamin A deficient diet together, the two rats which did not receive any light treatment until the fifth week of the experiment developed the typical eye lesion within 6 weeks. The only rat which escaped was No. 446, which received ultra-violet light from the beginning of the experiment till the ninth week. It actually increased in weight after the seventh week and never developed the typical eye lesion. It died after 20 weeks of pneumonia and enteritis. This is the first time that we have observed a rat from a vitamin-underfed stock to survive more than 10 weeks of vitamin A deficiency without developing the typical eye lesion or septic glands. The usual period is 6 to 7 weeks. Table II shows how the number of platelets in this rat was maintained at an almost normal level for the first 7 weeks of the experiment while the rat was receiving 5 minutes' exposures to ultra-violet light daily. The same table shows that rat 447 of the same litter had developed a profound thrombopenia as early as the fourth week. As the result of exposure to ultra-violet light, which began after the fourth week, the thrombopenia was arrested, and, in fact, the platelets showed a slight increase; xerophthalmia set in during the sixth week. The third rat (No. 448) developed the eye lesion in the fifth week—that is to say, within a week after the light treatment had begun—and died in the seventh week.

We find, then, that vitamin A and light have a similar effect in so far as the blood-platelets are concerned, although it does not necessarily follow that this effect is produced in the same way; both the intake of abundant vitamin A by the mouth and the application of light to the whole animal lead to an increase in the number of platelets, while in the absence of vitamin A from the food, or in the absence of light, the number of platelets diminished. But there is this important difference between these two agencies—vitamin A and light—that in the absence of vitamin A from the food the animal cannot live; sooner or later its health obviously declines, the intestinal mucous membrane atrophies, the animal ceases to grow, and eventually bacterial infections set in, which are usually localised either in the eyes or in the lymph-glands. None of these changes appear in animals growing up in darkness as long as they receive even a small supply of vitamin A. But such animals, when subjected to vitamin A deficiency, decline in health more rapidly than rats brought up in a well-lit room. This difference is brought out most distinctly when the rats have been kept on a diet not rich in vitamins. Thus three rats from a litter born in a dark room (13 . 11 . 22) and kept in this room on our ordinary vitamin-poor laboratory diet until they had reached a weight of 60 gm., 70 gm. and 80 gm. respectively, were compared with three rats 4 weeks younger (born 14 . 12 . 22) and weighing 50 gm. each, from a litter kept in our well-lit animal room on the same diet. Both sets of rats were placed on a vitamin A free diet and kept, the one set in the dark room, the other set in the ordinary animal room. Two rats from the "darkness" litter developed xerophthalmia after 4 weeks and the third rat after 5 weeks, the three rats from the control "light" litter only after 7 weeks, although they were actually younger. This experiment was repeated with the same result. This difference between litters kept in light and in darkness is less distinct when the rats are kept on a diet rich in vitamins previous to the deficiency period. The stimulating effect of vitamin A on platelets and the functional development of the intestinal mucosa counteracts the atrophic effect of darkness on the platelets. Thus three rats from a "darkness" litter kept on such a vitamin-rich diet have been kept in a dark room for 19 weeks on a vitamin A free diet. Three rats of the same age from a "light" litter were kept as control on the same diet but in the well-lighted animal room. One rat of the "darkness" litter developed the eye lesion and septic glands in the neck after 7 weeks. This is the first time that we have observed a rat from a vitamin-rich stock to develop the typical infections after so short a period of vitamin A deficiency. The other two and the three control rats of the "light" litter remain, during 17 weeks of vitamin A deficiency, without any obvious sign of localised infections, and, in fact, looked quite normal. The typical eye lesion then made its appearance in all five rats within 10 days.

There is some indication in our stock of rats kept on our vitamin-poor laboratory diet that there are seasonal variations in their resistance to vitamin A deficiency. As a rule such rats develop the typical eye lesion with great regularity after 6 to 7 weeks of vitamin A deficiency. The experiments are usually carried out in sets of three rats kept in one cage, and if the eye lesion appears first in one rat it is, as a rule, within 7 to 10 days that it appears in the other two rats. It has happened repeatedly, however, that the eye lesion appeared in a whole set of three rats with exceptional rapidity 4 to 5 weeks afterwards. In going back over the protocols it was found that this occurred only in experiments carried out between December and March. This statement refers only to rats kept on our vitamin-poor laboratory diet. Our animal room, which is situated on the top of the building, is exceptionally well lighted, receiving light from large windows along two sides of the room and also from the top. It is kept warm at a fairly constant temperature day and night throughout the winter, so that cold can be excluded as the determining factor. Although not every set shows this diminished resistance to vitamin A deficiency during the winter months, the experimental results with rats kept in darkness suggest that the seasonal variations in the resistance to vitamin A deficiency are related to variations in the amount of daylight.

While absence of light diminishes the resistance of rats to vitamin A deficiency, the application of ultra-violet light increases their resistance to this deficiency. This was shown clearly by Miss Hume (1922), and by Goldblatt and Soames (1922), who studied the effect of light in vitamin A deficiency with the object of obtaining evidence as to the curative action of light in rickets. They found that rats kept on a vitamin A free diet will live longer if receiving exposures to ultra-violet light at the same time. In the end, however, they also decline in health and develop the typical symptoms of vitamin A deficiency. They conclude, therefore, that the action of light cannot be explained by assuming that there is a photosynthesis of vitamin A in the cells of the skin. Powers, Park and Simmonds have obtained similar results.

MODE OF ACTION OF LIGHT.

Two years ago we drew attention to the surprising fact that vitamin deficiency and radiations from X-rays and radium produce similar effects (Cramer, Drew and Mottram, 1921). Further investigations have established the truth of this generalisation. It may be noted as an interesting fact that very small doses of these powerful radiations have a stimulating effect on the very tissues the functional activity of which they inhibit if large doses are given. They produce in small doses a lymphocytosis and a transient thrombocytosis instead of the lymphopenia and the thrombopenia produced by large doses. It is therefore possible that light in large doses, especially ultra-violet light, may have an inhibiting instead of a stimulating effect. In this connection it is interesting and important to note that clinically it has been found that excessive exposure to sunlight produces a hæmorrhagic diathesis (Hausmann, 1923), which may in lung tuberculosis lead to hæmorrhages. Experimentally, Levy and Gassul (quoted from Clark, 1922) found in mice as the result of prolonged exposure to ultra-violet light great congestion in spleen, liver and lungs. In the lungs the congestion was so great that red corpuscles had actually passed through the capillary walls into the alveoli. We have pointed out that the similarity between the action of two such different agencies as radiations and vitamin deficiencies is not readily compatible with the generally accepted conception of the mode of action of vitamins which postulates that the life of every cell is dependent on the presence of vitamins. On the basis of that conception it is even more difficult to understand how a physical agent such as light should be able to counteract a dietetic deficiency. We have in a previous paper (Cramer, 1923) developed a different conception of the mode of action of vitamins, which allows of a ready explanation of this apparently paradoxical antagonism between light and vitamin A deficiency. Our view of the mode of action of vitamins may be briefly re-stated as follows:

MODE OF ACTION OF VITAMINS.

Vitamins are substances present in the food in variable quantities which have a drug-like stimulating effect on specific tissues, resembling in this respect the hormones of the endocrine organs. These specific tissues are in part dependent for their full functional development on the continued presence of these "food hormones," as the vitamins may be called, in the food, so that in the absence of these "food hormones" atrophic changes occur in these tissues. Thus in the absence of vitamin A the intestinal mucous membrane atrophies. A further atrophic change is the diminution in the number of blood-platelets. Quite recently an atrophic change similar to that affecting the intestinal mucosa has been described by Yudkin and Lambert (1923) as occurring in the para-ocular glands and accompanying the characteristic eye lesion. It is not necessary to discuss here how these lesions are related to each other. We will only point out that a similar change in the intestinal mucosa is produced by exposure to radium and X-rays, and the observations of Mottram (1923^2) show that the primary effect is a disturbance in the secretion of mucus by the epithelial cells, which allows the intestinal bacteria to come into intimate contact with the mucosa. A similar deficient secretion of mucus occurs in vitamin A deficiency, and in a recent paper by one of us we published a figure of a mucous gland of the cæcum of a rat in vitamin A deficiency filled and distended with bacteria while showing an almost complete absence of goblet cells. Under the condition of our experiments, where the rats are kept in wooden boxes with a perforated zinc lid and are therefore not exposed to any great extent to the direct effects of daylight, as they would be, for instance, in a wire cage, the atrophy of the intestinal mucosa and the thrombopenia proceed *pari passu*. These lesions are the cause of the general symptoms of vitamin A deficiency—namely, cessation of growth, invasion of bacteria and diminished resistance to certain types of bacterial infections—and the rate at which these lesions develop determines the onset of the general symptoms. Of these two lesions, one, the thrombopenia, can be counteracted by exposure to light, which stimulates the production of platelets. It follows, therefore, that any condition which tends to diminish the number of platelets—such as absence of light—when applied before the animal is subjected to vitamin A deficiency, must handicap the organism in its reaction against the deficiency and hasten the decline. Conversely conditions which stimulate the production of platelets—such as exposure to light—must assist the organism in resisting the general effects of vitamin A deficiency and delay the decline. By thus referring the effects of vitamin deficiency to specific pathological lesions, it is possible to give an explanation of the apparently paradoxical antagonism between two such different agencies as light and a dietetic factor.

We have in a previous paper made the suggestion that the blood-platelets represent a defensive mechanism against bacterial infection, and given evidence from our own observations as well as from those of other workers in support of this view. The fact that a factor such as light, which stimulates the production of platelets, tends to delay the onset of bacterial infections in vitamin A deficiency, is further evidence in support of this view. It is also interesting to note that Yudkin and Lambert quote Wason and Smith as having found a diminution in the bactericidal titre of the serum of deficiently fed animals.

ON THE ORIGIN OF PLATELETS.

The problem of the manner in which light produces its effect in increasing the number of platelets is bound up with the problem of the origin of platelets, of which latter problem we know unfortunately very little. If the platelets are formed exclusively in some internal organ—and it is held by some observers that they are formed by the megakaryocytes of the bone-marrow—then the action of light on platelet formation, which can only affect the cells of the skin or the underlying capillaries and lymphatics, can only be explained by an indirect photosynthetic effect. We would then have to assume that these peripheral cells under the influence of light form a chemical substance which passes into the blood and stimulates the production of platelets in sites such as the bone-marrow which are remote from the direct action of light. In order to obtain information on the origin of platelets we have examined the bone-marrow both in smears and in paraffin sections from normal rats, rats suffering from a vitamin A deficiency and rats recovering from vitamin A deficiency. The material was fixed in sublimate acetic acid, stained with Giemsa, and differentiated with mixtures of acetone and xylol in varying proportions. We have never been able to obtain evidence which would indicate the formation of platelets from the megakaryocytes. In fact the bone-marrow was always remarkably poor in platelets. On the other hand the spleen is frequently very rich in platelets, which lie scattered through the parenchyma of the spleen. In one instance a section through an artery and a vein lying side by side showed the vein crowded with platelets while the artery showed very few. Veins from lymph-glands in the subcutaneous tissue have also often been found to contain numerous platelets. In some preparations from rats recovering from vitamin A deficiency under the influence of cod-liver oil, the veins show a sprouting of the cytoplasm of the endothelial cells with platelets lying embedded in the cytoplasm. Platelets are also very numerous in the lymphatics of the mesentery of the rat. These appearances, which are put forward at present as representing merely the results of a preliminary investigation, suggest that platelets are formed by the endothelial cells of lymphatics and blood-vessels. This view of the origin of platelets agrees with the findings of Bedson (1922) that antiplatelet serum, which is highly specific for platelets, has also a toxic effect on the endothelium of the blood-vessels. If platelets have a general origin of this kind, then the effect of light might be attributed to a local stimulating action of light on the protoplasm of the endothelial cells in the peripheral capillaries and lymphatics. Light is, however, not essential for the formation of platelets. For platelets are present in considerable numbers in the blood of the embryo.

Variations in the number of platelets are bound to have an effect on the chemical composition of the blood. For platelets which represent an extremely sensitive and delicate type of protoplasm must be conceived as constantly undergoing disintegration and pouring their contents into the plasma. This may account for the chemical changes which have been found to occur in the blood as the result of exposure to light.

SUMMARY.

Rats born and kept in darkness frequently show a diminution in the number of blood-platelets as compared with rats kept on the same vitamin-poor diet in a well-lighted room. This thrombopenia is sometimes associated with a definite anæmia. Exposure of such animals to a mercury vapour lamp increases the number of blood-platelets to normal, and, if an anæmia was present, increases the number of red cells. Rats kept in darkness do not exhibit intestinal lesions such as are found in vitamin A deficiency, nor do they develop the bactericidal infections (xerophthalmia, septic lymph-glands) characteristic of that deficiency. They do, however, develop these conditions sooner when placed on a vitamin A deficiency diet than rats kept in a well-lighted room.

Rats kept on a vitamin A free diet develop a progressive thrombopenia and an atrophy of the intestinal mucosa. Exposure to a mercury vapour lamp if applied from the beginning of the deprivation of this vitamin, delays the

thrombopenia. If applied after a thrombopenia has set in, it will increase the number of platelets. Such exposures to light, however, do not prevent the eventual onset of the atrophy of the intestinal mucosa which results from the vitamin A deficiency.

The conclusion is drawn that light is a stimulus to the formation of blood-platelets. This explains the partial antagonism between vitamin A deficiency and exposure to light: light can counteract the one lesion—thrombopenia—produced by vitamin A deficiency, but cannot prevent the eventual atrophy of the intestinal mucous membrane. Exposure to light can therefore delay but cannot prevent the eventual cessation of growth and the eventual onset of bacterial infections resulting from the vitamin A deficiency. Absence of light accelerates the onset of these conditions. Light is, however, not an essential condition for the formation of platelets, and its absence can be counteracted so far as the platelets are concerned by an abundant supply of vitamin A.

The fact that conditions which stimulate the production of platelets will delay the development of certain types of bacterial infections is further evidence in support of the view that platelets represent a mechanism against bacterial infection. The mode of action of light and the origin of platelets are discussed.

REFERENCES.

Bedson, S. P.—(1922) "Blood-platelet Anti-serum, its Specificity and *Rôle* in the Experimental Production of Purpura," *J. Path. & Bact.*, **25**, 94.

Bernhard, O.—(1917) 'Sonnenlichtbehandlung in der Chirurgie.' Stuttgart (Enke).

Clark, J. H.—(1922) "Physiological Action of Light," *Physiol. Rev.*, **2**, 285.

Cramer, W.—(1922[1]) Discussion on "The Physiological Action of Light," *Lancet*, **2**, 1120.—(1922[2]) "On Vitamin Underfeeding," *Brit. J. Exper. Pathol.*, **3**, 298.—(1923) "On the Mode of Action of Vitamins," *Lancet*, **1**, 1046.

Cramer, W., Drew, A. H., and Mottram, J. C.—(1921) "Similarity of Effects Produced by Absence of Vitamines and by Exposure to X-rays and Radium," *Lancet*, **1**, 963.—(1922) "On Blood-platelets: Their Behaviour in Vitamin A Deficiency and After Radiation, etc.," *Proc. R. Soc.*, Ser. B., **93**, 449.

Fabricius-Möller, A.—(1922) 'Experimentelle Studier ooer Hæmorrhagisk Diathese.' Copenhagen. See also *Compt. rend. Soc. de biol.*, 1922, **87**, 759.

Goldblatt, H., and Soames, K. M.—(1922) "The Effect of Radiation with the Mercury Vapour Quartz Lamp," *Lancet*, **2**, 1321.

Hausmann.—(1923) 'Grundzüge der Lichtbiologie and Lichtpathologie.' Berlin (Urban and Schwarzenberg).

Hume, H.—(1922) "The Effect of Radiation with the Mercury Vapour Quartz Lamp," *Lancet*, **2**, 1318.

Mottram, J. C.—(1923[1]) "Some Effects of Exposure to Radium on the Blood-platelets," *Proc. R. Soc. Med.*, **16**, Section of Pathol., p. 9.—(1923[2]) "Some Effects of Exposure to Radium upon the Alimentary Canal," *Proc. R. Soc. Med.*, **16**, Section of Electrotherap., p. 41.

Yudkin, A. M., and Lambert, R. A.—(1923) "Pathogenesis of the Ocular Lesions Produced by a Deficiency in Vitamin A," *J. Exp. Med.*, **38**, 17.

DIPHTHERIA TOXOID AS AN IMMUNISING AGENT.

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IN a previous paper (Glenny, Allen and Hopkins, 1923) methods have been described for testing diphtheria toxin-antitoxin mixtures for use in active immunisation against diphtheria. By means of the methods there described it is now possible to determine the relative antigenic values of mixtures of toxin and antitoxin of varying strength and constitution, and of modifications of toxin. An ideal immunising agent is one that presents the highest antigenic strength but does not cause any injurious effects. The object of this present paper is to show what work has been done towards producing an ideal immunising agent against diphtheria.

Most of our knowledge of the relative immunising values of different toxins has been obtained from the routine immunisation of horses for the large scale production of diphtheria antitoxin. Experience has shown that the immunising value of a batch of toxin depends upon its specific antigenic value in relation to its toxicity. In this connection, by toxicity is meant not only specific toxicity, *i. e.* the number of minimal lethal doses of diphtheria toxin present, but also non-specific toxicity due mainly to broth constituents. When we are dealing with toxin-antitoxin mixtures for human use it is necessary also to consider the amounts of horse-serum present when given to serum sensitive subjects, and also of the "pseudo" constituent in the toxin when the mixture is used to immunise a "combined" (pseudo and positive) Schick-reactor. The specific antigenic value of a toxin or of a toxin-antitoxin mixture depends upon the number of free binding units, whether of toxin or toxoid. For the purposes of this paper the term "toxoid" will be used in its general sense for any modification of toxin that will still combine with antitoxin but is no longer fatal to guinea-pigs. That toxoid is of equal specific antigenic value to toxin has long been known; in 1904 one of us produced diphtheria antitoxin containing over 1000 units per c.c. from a horse injected only with diphtheria toxin so modified that the M.L.D. for a guinea-pig of 250 gm. weight was 0·3 c.c. and the L 0 dose 0·5 c.c.; a few years later similar high value antitoxin was obtained from a horse injected with modified toxin, of which 5·0 c.c. would not kill a guinea-pig. The immunising value of modified toxin rich in toxoid is greater than that of toxin because, while the specific antigenic value remains the same, the specific toxicity is reduced or absent. When we are dealing, however, with hyper-immunisation, specific toxicity is of less importance, owing to the presence in the animal of sufficient circulating antitoxin to neutralise most of the specific toxin. Yet it is interesting to note in passing that a small local injection is not all neutralised by circulating antitoxin, although the total antitoxin present in the animal may be enough to neutralise many thousand times the amount of toxin injected. The Schick reaction in man is a typical example of this; a man with five litres of blood containing $\frac{1}{100}$ unit of antitoxin per c.c. gives a Schick-positive reaction when injected with that amount of toxin which would be completely neutralised by $\frac{1}{1000}$ unit of antitoxin, *i.e.* $\frac{1}{50000}$ of the total circulating antitoxin in the patient. Again, immunised horses containing, say, 30 litres of blood have developed diphtheria paralysis after an injection of toxin that could be completely neutralised by 1 c.c. of their blood.

The use of modified toxin in toxin-antitoxin mixtures for human use enables a far greater number of binding units, *i. e.* a greater specific antigenic strength, to be presented without any increase in specific toxicity. It may be possible shortly to use toxin so modified that it will be completely non-toxic without the addition of antitoxin. This would constitute a marked advance in the prevention of diphtheria; the presence of horse-serum in a toxin-antitoxin mixture might possibly cause reactions in serum-sensitive subjects, and possibly sensitise others not already sensitive, although no worker has as yet found evidence of sensitisation by such small doses of serum in a human subject. The use of modified toxin, however, reduces the amount of serum necessary in a mixture, because such modified toxin need not be fully neutralised; only sufficient antitoxin need be added to reduce the residual toxicity below a certain level.

The cause of the variation in non-specific toxicity of different batches of toxin is not known, nor is there any convenient test beyond that of large scale immunisation of horses. In our experiences two batches of toxin may have identical values for M.L.D., L 0 and L +, and so presumably have the same specific antigenic values, and yet differ so markedly in their true immunising property that one may be regarded as almost worthless and the other as extremely good. It is not yet known whether non-specific toxicity is due entirely to absolutely non-specific broth constituents or whether bacterial protein may play some part. In toxin-antitoxin mixtures intended for human use dilutions of the mixtures must lessen the non-specific toxicity.

The original American standard for specific toxicity of a toxin-antitoxin mixture was that 1 c.c. should cause no ill-effects in a guinea-pig, while 5 c.c. should kill in 10 days or more. Later the specific antigenic value was increased by using mixtures with more free toxin, and it was found that mixtures were safe for human use if 1 c.c. did not kill guinea-pigs in less than 10 days. A still greater improvement was made by using dilutions of more toxic mixtures. We, however, have endeavoured to increase the number of binding units in mixtures without making them any more toxic, and have continued to accept the early American standard of toxicity.

The modification of toxin used in the experiments recorded in this paper was prepared by adding 0·1 per cent. formalin to diphtheria toxin and exposing it for 4 weeks at a temperature of 37° C. before removing to the cold room. The original values of the toxin showed an M.L.D. of 0·0025 c.c.; L 0, 0·15 c.c.; and L +, 0·2 c.c. After treatment the M.L.D. was 0·2 c.c.; the L 0, 0·3 c.c.; and the L +, 1·0 c.c.

The routine method now adopted by us for testing antigenic values has already been described by us (Glenny, Allen and Hopkins, 1923). This method consists in injecting guinea-pigs with a single dose of the toxin-antitoxin mixtures or toxin under test, resting the animals for 3 weeks, and then injecting at weekly intervals with Schick doses of toxin until the animals fail to show a reaction. The number of Schick doses so given, until the animal is sufficiently immune to show no reaction, is an index of the antigenic value of the material under test, and is now termed by us the "immunity index." Thus, if a guinea-pig was injected with 1·0 c.c. of a certain toxin-antitoxin mixture, and 3 weeks later the first Schick injection gave a positive reaction, and the following week the second Schick injection showed no reaction, then the immunity index for 1·0 c.c. of that mixture would be recorded as 2.

Occasionally in experimental work we have endeavoured to determine whether a substance has any value as an antigen to diphtheria. If the antigenic value is very low many Schick injections will be needed before the immunity of the test-animal is raised to the Schick-negative level; if the substance under test has no antigenic value, the immunity index will be the same number of Schick doses of diphtheria toxin that would need to be given at weekly intervals to a normal guinea-pig in order to raise its immunity to the Schick-negative level. It is necessary, therefore, to know the effect of weekly injections of Schick doses of toxin upon normal guinea-pigs.

In all experimental work on animals we have adopted as the Schick dose of toxin the amount that is just neutralised by $\frac{1}{1000}$ unit of antitoxin. This level was adopted because it represented the binding-unit content of $\frac{1}{50}$ M.L.D. of a well-matured toxin used in our first experiments, and, as we have already pointed out (Glenny and Allen, 1922), the level of immunity detected by a Schick test depends upon the number of binding units injected and not upon the toxin content alone. It was found that the number of weekly Schick doses necessary to immunise a guinea-pig varied according to the toxin used. Table I shows that a relatively fresh toxin, of which $\frac{1}{30}$ M.L.D. was equivalent to $\frac{1}{1000}$ unit of antitoxin, gave an average index of 31, while the shortest time in which a guinea-pig injected weekly with this dose became immune was 20 weeks. An old matured toxin, containing $\frac{1}{50}$ M.L.D. to the Schick dose, gave an average index of 12, while the lowest record for the toxin was 9. With the modified toxin one guinea-pig, when given only $\frac{1}{200}$ M.L.D. each week, was sufficiently immune to give no reaction to the sixth weekly injection, while the majority were immune by the time they received the seventh injection.

TABLE I.

Toxin number.	Description.	Volume injected.	Fraction of M.L.D.	Average index.	Lowest index.
J. 3284	Fresh toxin	0·0001 c.c.	$\frac{1}{30}$	31	20
J. 1915	Old toxin	0·0004 c.c.	$\frac{1}{50}$	12	9
Y.M.B. 101	Modified toxin	0·001 c.c.	$\frac{1}{200}$	7	6

If so few injections of so small an amount of "toxoid" will immunise a guinea-pig, we may hope in the course of time so to improve the methods of diphtheria prevention that a single dose of modified toxin will act both as a Schick test and as an immunising agent.

The antigenic values of one or two injections of modified toxin acting as a primary stimulus were measured by the method already described, and the results are recorded in Table II.

TABLE II.

	No. of experiments showing an immunity index of—						
Dose.	1	2	3	4	5	Over 5.	Total.
Two injections of 0·01 c.c. .	—	4	2	—	—	1	7
One injection of 0·05 ,, .	—	15	9	5	5	5	39
,, ,, 0·1 ,, .	2	—	—	—	—	—	2
,, ,, 0·2 ,, .	1	1	—	—	—	—	2

The general condition of the test animals has a great influence upon the apparent immunity index of an antigen. A good antigen may occasionally show a poor index; thus in Table II two injections of 0·01 c.c. modified toxin have yielded a bad index once in seven experiments. A large number of tests have been made at different times upon the immunity index for a single dose of 0·05 c.c. because we have found that this is a useful critical dose, sufficiently effective as a stimulus to yield a very good index of 2 on many occasions, while at the same time the dose is not excessive. The minimal dose that will constantly yield a good index under good conditions will frequently yield a poor index if the test animals are subject to adverse conditions; the immunity index of a larger dose is not so easily affected. For this reason we use a dose of 0·05 c.c. of this modified toxin when testing the conditions affecting the production of immunity. The experiments quoted in the table refer only to those guinea-pigs that were not injected with other substances to affect the rate of production of immunity, but include all control animals and those of varying weight. Eight out of the ten experiments showing an immunity index of 4 or 5 occurred in a single group of animals injected at a time when feeding conditions were not good, and, consequently rate of growth was slow and infection rate high. The antigenic value of a single dose containing 0·05 c.c. of modified toxin is at least as good as that of the majority of batches of toxin-antitoxin mixtures which we have tested. Such a dose of unneutralised modified toxin is not more specifically toxic than a toxin-antitoxic mixture, contains from one-tenth to one-twentieth of the non-specific constituents, and in addition contains no horse-serum. Four guinea-pigs were injected with higher doses, and it will be seen from Table II that three out of four guinea-pigs injected with from ½ to 1 M.L.D. were Schick negative when first tested 3 weeks later, while the fourth animal was negative at the second test made 4 weeks after the primary stimulus.

The action of toxoid as a primary stimulus has already been described by one of us (Glenny and Sudmersen, 1921), when it was pointed out that "three weeks after a single dose of from 0·5 c.c. to 2·0 c.c. of an old formalinised toxin (L 0 dose about 1·0 c.c.; M.L.D., 5·0 c.c.), a number of guinea-pigs survived 2 M.L.D. of toxin without any local reaction." One guinea-pig that had received four injections of toxoid over a long period of time reached an antitoxic titre of 8·5 units per c.c.

In the preparation of toxin-antitoxin mixtures from unmodified toxin there is very little margin between mixtures that are too toxic and mixtures so over-neutralised that they are of very low antigenic value. With modified toxin the differential region between L 0 and L + is so great that there is a very large margin of safety in the preparation of mixtures.

TABLE III.

Dose injected: 0·1 c.c. of a mixture containing—	Number of experiments showing an immunity index of—						
	1	2	3	4	5	Over 5.	Total.
0·1 c.c. antitoxin (27 units) + 10·0 c.c. modified toxin (approx. L 0)	—	2	4	—	—	—	6
0·1 ,, ,, 14·0 ,, ,,	—	2	—	—	—	—	2
0·1 ,, ,, 20·0 ,, ,,	—	3	1	—	—	—	4
0·1 ,, ,, 25·0 ,, ,,	—	1	2	—	—	—	3
0·1 ,, ,, 30·0 ,, ,, (approx. L+)	1	6	—	—	—	—	7
0·1 ,, ,, 42·0 ,, ,,	—	2	—	1	—	—	3
0·1 ,, ,, 50·0 ,, ,,	—	—	2	—	—	—	2

Table III shows that mixtures may be prepared containing from 10 to 50 c.c. of modified toxin to the same amount (0·1 c.c.) of a certain antitoxin, and with all mixtures throughout this range the immunity index for 0·1 c.c. of the mixture is as good as that for 1·0 c.c. of the majority of the mixtures prepared from unmodified toxin. Several of the mixtures were tested at lower doses; thus the mixture containing 42 c.c. of modified toxin and 0·1 c.c. of antitoxin was injected in doses of 0·01 c.c. into 3 guinea-pigs; 2 gave an immunity index of 2 and 1 of 4. In this mixture the toxoid was only neutralised to the extent of about 20 per cent. The specific toxicity of the mixture was such that when diluted 1 in 10 the mixture would correspond with the U.S.A. standard for toxin-antitoxin mixtures, yet a high index is given when diluted 1 in 100.

The effect of modified toxin as a secondary stimulus to actively immune rabbits is shown by Table IV.

TABLE IV.

Rabbit.	Antitoxic value before injection in units per c.c.	Volume of modified toxin injected.	Maximum value after injection in units per c.c.	Time interval.
10	0·08	5·0 c.c.	12·0	8 days.
42	0·0005	0·05 c.c.	2·75	6 ,,
55	0·0005	0·05 c.c.	3·75	6 ,,
99	0·015	0·1 c.c.	1·75	11 ,,
120	0·0005	0·05 c.c.	0·04	8 ,,
121	0·0005	0·05 c.c.	0·03	6 ,,
148	0·0005	0·05 c.c.	9·0	10 ,,
151	0·005	0·05 c.c.	4·5	8 ,,

Further work is being carried out along the following lines:

(1) Purification of toxin by methods of concentration which increase the number of binding units per milligramme of nitrogen about 40 fold.

(2) Using toxin so further modified that it is not toxic even in large doses.

(3) Investigation of the spectrum of partial neutralisation in relation to antigenic values.

Our thanks are due to our medical colleagues Drs. O'Brien, Eagleton and Okell for the information that a mixture of modified toxin and antitoxin (that containing 20·0 c.c. of "toxoid" to 0·1 c.c. of antitoxin and diluted 1 in 10) has given exceptionally promising results when employed for human immunisation.

SUMMARY.

The value of a diphtheria toxin for immunising purposes increases as toxin changes into toxoid (or toxone).

Toxins can be changed into toxoid by the action of formalin.

Such modified toxin containing 1·5 M.L.D. per L 0 and 5 M.L.D. per L+ has been used for the production of toxin-antitoxin mixtures showing a high immunity index.

It is hoped by further reduction in toxicity and by concentration to produce an immunising agent of far greater efficiency than any yet employed for protection against diphtheria.

REFERENCES.

Glenny, A. T., Allen, K., and Hopkins, B. E.—(1923) *Brit. J. Exper. Pathol.*, **4**, 19.
Glenny, A. T., and Allen, K.—(1922) *Lancet*, **1**, 227.
Glenny, A. T., and Sudmersen, H. J.—(1921) *J. Hyg.*, **20**, 176.

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THE STANDARDISATION OF TUBERCULIN.

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IN a previous communication (Eagleton, 1921), a description was given of the method employed at this laboratory for the standardisation of tuberculin.

Briefly stated, this method depends on the specific action of tuberculin on tuberculous animals, the potency of different specimens being compared in this respect with that of a standard tuberculin. A standard was fixed originally by Ehrlich, who showed that tuberculins of the strength adopted were efficient for human and veterinary use.

In 1912 Ehrlich presented to these laboratories a sample of his official Frankfort standard. Upon this our own standard was based, and this has been checked with a sample received from Prof. Kolle of Frankfort in 1921 (*vide* p. 301, Table VII).

The test in fact is essentially that devised by Koch and still used with slight modifications at Frankfort and other places. (Koch, 1891; Otto, 1904; Reeser, 1908; Loewenstein, 1913; Otto and Hetsch, 1921.)

Since our last communication the method has been improved and simplified, large numbers of samples have been tested and re-tested, and several hundred animals employed. We feel that this experience should prove of value to others, and for this reason we propose in this paper to give a *résumé* of the method, a careful consideration of the details and difficulties encountered, and finally a summary of a few of the more important results obtained during the last three years.

DESCRIPTION OF THE TEST.

Sensitisation of animals.—A batch of guinea-pigs is infected with a standardised emulsion of living tubercle bacilli, 0·5 c.c. being injected into the front and inner aspect of the right thigh, deep to the adductor muscles.

The batch is examined week by week, and when the right inguinal glands are easily palpable we proceed to—

The preliminary intracutaneous test.—About the third or fourth week after infection two animals are selected, a small white portion depilated, and into the skin of each three doses of standard tuberculin injected (0·2 c.c. of dilutions 1/500, 1/1000, 1/2000). This test is repeated weekly until, with the highest dilution of the series, there is obtained next day a strongly positive reaction. We then proceed to—

The main intracutaneous test.—Dilutions of the standard, 1/1000 to 1/8000, are injected as above into the skin of at least two animals, and into corresponding areas of the same animals are injected the same dilutions of the specimen under test. From the severity of the reactions so produced the strength of the sample relative to the standard can be determined. This method is accurate and simple. It is our practice, however, to check all its findings by the—

Subcutaneous method.—This, the original Koch method, is performed as follows:

Preliminary subcutaneous test.—When the animals are sensitive as judged by the intracutaneous test (*vide supra*) this test may be undertaken. One animal of the batch with easily palpable glands is injected with 0·25 c.c. of standard tuberculin subcutaneously. If it survives, or dies at a greater interval than 24 hours, the test is repeated the following week. As soon as 0·25 c.c. kills an animal, three further animals are selected and injected with 0·25 c.c., 0·1 c.c. and 0·1 c.c. of tuberculin respectively. The animal injected with 0·25 c.c. and *one* of those injected with 0·1 c.c. should die overnight. Having obtained this, our "standard reaction," we proceed to—

The main subcutaneous test.—If in the preliminary test the "standard reaction" has been obtained, we proceed on the next day as follows: Each sample that has been found by the main intracutaneous test to be equal to the standard is injected into two animals (0·1 c.c into each). Samples found by the intracutaneous test to be higher or lower than the standard are employed in suitable doses.

The "standard test" consists, then, of a combined intracutaneous and subcutaneous method. The diagram (Table I) shows schematically the testing by this method of a sample which proved equal in strength to the standard.

TECHNICAL DETAILS.

It is not to be thought that the test is essentially a difficult one. We have found, indeed, that with careful attention to detail each factor in the test may be controlled sufficiently to ensure constancy of result.

There are three main factors involved—the animals, the inoculum for sensitisation and the method of dilution and injection.

Animals.

Sufficient animals must be injected to provide for loss from natural causes and to ensure survivors for the cutaneous and subcutaneous test. We find 60 a convenient number for a batch, of which at least one-third should have white or cream areas of skin. They should be young animals and as nearly as possible of the same weight (250 gm.). The use of older animals involves differences in sensitivity owing to certain members of the batch becoming pregnant.

The animals may be "controls" and other survivors from toxin-antitoxin titrations; such "used" animals may be included with fresh stock to form a batch for sensitisation. Previous injections of serum or materials other than tubercle bacilli or their products appear to have no untoward influence on the constancy of the test.

The cutaneous sensitivity to tuberculin of certain animals is apparently increased by a previous injection of horse-serum, so that we often find certain individuals in a batch reacting to a smaller dose of tuberculin than their fellows. This statement, however, only applies to the cutaneous reaction, and the subcutaneous test is not affected. Moreover, animals used for the intracutaneous and von Pirquet test can be kept and used for the subcutaneous injection, provided they are in good condition at the time of the latter. Survivors from the subcutaneous test come, however, into a different category.

Most animals recover from a sublethal dose in five days, and regain their full sensitivity to subsequent injections. This is not, however, invariable, inasmuch as certain animals are rendered refractory for a shorter or longer period, and this variability appears to depend on some idiosyncrasy of the animal. It is noted, moreover, that the more seriously an animal is "shocked" by the first dose, the greater the chance of its remaining insensitive for an abnormally long spell. Thus while animals which have survived one subcutaneous dose may generally be used again after an interval of five days, the results of such tests need great care for their interpretation.

It will be seen in Table II that two animals were unaffected by large doses of sample A. The fact that they subsequently succumbed to the minimal lethal dose of the standard tuberculin proved that it was not due to lack of sensitivity that they failed to respond, but to the weakness of sample A. This reinjection of animals which have survived the first test can thus be employed with advantage as a check on their original sensitivity. Table III, on the other hand, shows the care needed in the interpretation of results. Refractory animals may show merely a large local reaction, with very little, if any, general disturbance. The animals of batch 64 are seen to be of "standard sensitivity," while those of batch 63 are slightly more sensitive. The survivors from these tests were mixed together and used for a second subcutaneous test seven days later. The results are consistent except in the case of three animals, two of whom were seedy but survived when we know they should have died with these doses of sample A. A third animal, not shown on the chart, died from some cause other than the tuberculin injection.

Typical and atypical deaths.—Before we pass on to consider the "atypical" deaths and their chief causes, it is well to emphasise what we mean by a "typical" death (designated in all our work by †).

The animal must die within 24 hours of the tuberculin injection, and show the standard lesions produced in the tuberculous by tuberculin—local œdema, congestion and œdema round the glands affected by the tuberculous process, congestion of any visceral foci that may be present, engorgement of the spleen so marked that in a well-defined case this organ is purplish-black in colour, œdema of the head of the pancreas of such an extent that the duodenal loop of intestine appears to bound a solid mass of jelly. Of these signs the most constant is undoubtedly the condition of the spleen.

Atypical deaths, signified in our tables as X, are of two kinds. The first kind may be described as a late or atypical result of the tuberculin. Thus we frequently find that at the minimal lethal level of a tuberculin one animal may die in a typical manner, while the other dies at the end of 48 hours with less obvious signs of tuberculin reaction (*vide* Table I). Such late deaths are frequent in animals rendered partially refractory by a previous dose of tuberculin. They are also frequent in batches of animals that are dying of Gaertner and other non-specific infections. No animal should be used for the subcutaneous test without a satisfactory examination of its physical fitness. The better in health the tuberculous guinea-pig is, the more constant and typical is its reaction to tuberculin.

Tuberculous animals under good conditions should show a regular increase in weight; they should be active and their muscles firm; their coat should be smooth and glossy and their hair should not fall out. Their appearance indeed should be that of a normal animal, and should a batch of guinea-pigs appear seedy and weak, etc., it is quite certain that some other factor than the tuberculous process itself is at work.

We do not intend to discuss here the chief epidemics with which we have to contend. Suffice it to say that they are chiefly due to two organisms, one associated mainly with enteritis and nervous phenomena—a species of *B. enteriditis* of Gaertner—and a second giving rise to pneumonia, "snuffles" and enteritis, this organism being indistinguishable culturally from *B. fæcalis alkaligenes*. These epidemics are mainly responsible for the second kind of atypical death, which has no connection with tuberculin injection. When once an epidemic due to either or both of these germs is established, very little can be done to control the disaster, but the following palliative measures may be tried. Animals falling sick are immediately removed from the batch; if the death-rate rises to above one or two per day out of a batch of sixty, the batch is carefully examined, all seedy animals removed and the batch transferred to a clean run. Any deficiencies in food supply are remedied as far as possible (*vide infra*). In spite of this, some batches of animals are so hard hit that out of sixty none survive for test purposes.

If, then, an epidemic be established we have little hope of saving many animals of an affected batch.

Our knowledge of the ætiology of these epidemics enables us, however, to take preventive measures. The influence of diet on epidemics among small animals has been noted by many workers, and the value of green food-stuffs was well shown by Glenny and Allen in a paper read before the Pathological Section, Royal Society of Medicine, in November, 1921.

We have ourselves examined culturally animals dying in epidemic and non-epidemic periods, and have found that many apparently healthy animals, even in non-epidemic seasons, carry organisms of the Gaertner or *fæcalis* type in the gut or the ileo-cæcal and mesenteric glands. Of 68 tuberculous animals submitted to careful bacteriological examination after death 30 per cent. died of generalised Gaertner infection, 3 per cent. carried these organisms in the ileo-cæcal and mesenteric glands, *i. e.* were chronically infected, and 10 per cent. in the intestinal contents, and so may be regarded as carriers.

The organisms responsible for the epidemics may then be regarded as always present, and it is upon the second factor, diet, that we must concentrate.

Tuberculous guinea-pigs should receive a larger ration than non-tuberculous animals, with plenty of green food or swedes; a supply of water is an advantage in hot weather.

They should be kept in large airy runs, on dry bedding (*e.g.* wood-shavings); in cold weather artificial heat must be provided. Given such an environment epidemics are unlikely to occur.

We have dwelt at considerable length on the difficulty of maintaining the health of tuberculous guinea-pigs. For long periods there may be no sign of trouble, but, should the irksome details of thorough routine supervision be relaxed, surely as night follows day disaster will occur.

The Inoculum for Sensitisation.

For this purpose we still use the culture sent us by Dr. Stanley Griffiths. This is a human strain which we have once only passed through a guinea-pig. It is maintained on special medium, which consists of Dorset's egg medium to which is added 5 per cent. glycerine. A three to 5 weeks growth is emulsified by grinding in sterile 0·9 per cent. saline, and standardised to contain 1 mg. of tubercle bacilli, weighed wet, per c.c. Care should be taken that the inoculum is as homogeneous as possible, and it should be shaken thoroughly before being used. We have also used emulsion of tuberculous animal tissue, as practised by Schroeder, at Washington (Schroeder and Brett, 1919), and we generally have one or more tuberculous rabbits available in case by some accident there is no suitable culture for use when required. Such animal emulsions are regular in their infecting power, but generally act slowly, so that the animals take a longer period than usual before they are fully sensitive.

With our standard strain a suitable animal tissue can be obtained by injecting 10 mgm. intravenously into a rabbit and killing the animal 10 days to a fortnight later. The lungs are then homogeneous and solid, and though few tubercle bacilli can be seen, the material is quite satisfactory for infecting the batch.

Method of Dilution and Injection.

With regard to dilution of the tuberculin, there is one point that may be emphasised. Owing to the high glycerine content (5 per cent. for P.T.O. or T.O.A. and 50 per cent. for P.T. or T.) these preparations are very viscous. To avoid any chance of carrying over material from mixtures of higher or lower concentrations, separate pipettes should be used for making each dilution, and great care should be taken to obtain thorough mixture.

Dilutions may be kept in the cold room till next day, but we prefer to use them as soon as they are prepared.

Of the subcutaneous injection we need say little. The injection should be truly subcutaneous and not intraperitoneal. For not only is the minimal lethal dose slightly different in the latter case, but the *post-mortem* appearances are less distinctive.

With regard to the *intracutaneous method* there are several points that require attention.

Preparation of animals.—Each animal selected must undergo a three-fold examination: it must be in good general condition, as was emphasised for the subcutaneous test (animals that are thin, even if showing no other definite signs of disease, are of no use for this test); it must be infected to the same degree as the rest of the batch, as judged by the size of the inguinal glands on the infected side; and lastly, it must have a sufficient area of white or cream colour to allow for the number of injections required.

The areas of skin chosen are first clipped and then depilated by means of a calcium suphide paste, prepared as follows:

To slaked lime is added sufficient water to make a creamy paste. To this add 2 per cent. of its volume of glycerin. Pass H_2S through the paste for 20 minutes to half an hour. Keep in a well-stoppered bottle.

There are one or two details to which attention must be paid. In the first place, only the part necessary should be depilated, the paste should not be left on too long and should be thoroughly removed after the operation, otherwise, owing to the irritant nature of the application, the skin may become inflamed and scratched. The animals should be well dried and kept warm.

Method of injection.—The injection, must be made into and not beneath the skin, the needle inserted bevel upwards, being carried to the same depth each time. We find it convenient to use rustless steel needles and all glass syringes.

Practice is needed if uniformity of injection is to be obtained, and allowance must be made for the variations in thickness of skin in different parts. Thus the skin of the back is very much more tough and thick than that of the abdomen and the depth of the injection must vary accordingly. The quality and size of the resulting lesions are also largely determined by the tension of the surface layers at the place injected, the skin of the abdomen, flanks and back all differing in this respect. For this reason the series of dilutions of tuberculins under test must be injected across the width rather than along the length of the animal, as follows:

```
        A.                          B.
      Head.                       Head.
                                  9  5  1
    4  3  2  1                   10  6  2
    8  7  6  5                   11  7  3
12 11 10  9                      12  8  4
      Tail.                       Tail.
```

A shows the correct way of placing injections. B shows the incorrect method.

1, 2, 3, 4 = 1/8000, 1/4000, 1/2000, 1/1000 standard.

5, 6, 7, 8,
9, 10, 11, 12, } corresponding dilutions of two samples under test.

The injections should not be made high up on the neck (anterior to the forelimbs), as this part has been found to give very small reactions in certain instances. Injections should not be made near the axillary or inguinal folds, as the material drains away and a smaller reaction results. This is especially well shown in the right inguinal region, where the tuberculous glands can be seen inflamed and sometimes connected with the lesion nearest to hand by a band of red.

There should be a space of about ¾ inch between the injections.

After injection the animal should be returned to a warm place, and if it show excessive signs of cold or shock, a hot bran mash may be given.

Readings are taken at 24 and 48 hours after injection. In most cases the reactions are well seen the next day. In some animals they develop more slowly, and in all cases it is better to read them after 24 and 48 hours.

The lesions vary slightly in size and intensity with the animal employed, and for this reason it is necessary that the dilution of the standard tuberculin be repeated on every animal used. Each animal is "read" independently, the strongest reaction on it being $++$, and the remainder graded from this $\pm\pm$, $+$, $\pm$, $\underline{\pm}$, $-$.

Owing to these differences in response we are unable to give an absolute value to any sample, but only its activity relative to the standard.

Two main fallacies may give rise to faulty readings: (1) errors in injection, leaks, etc.: (2) injections into unsuitable areas of skin (*vide supra*) or into old scar-tissue. An animal which has been carefully injected can be read by a novice; an animal which has been carelessly injected will defeat the expert.

The intracutaneous and von Pirquet reactions.—In our previous communication on this subject we showed that by means of the von Pirquet test we could obtain a guide for the subcutaneous test and so save time and animals. During the last three years we have developed the intracutaneous test and gradually abandoned the von Pirquet. All liquid tuberculin made in these laboratories, for human or veterinary use, is tested by the standard method (intracutaneous test checked by the subcutaneous test). Tuberculin that is to be used for von Pirquet or similar diagnostic tests is, in addition, submitted to the von Pirquet test.

For this we still adhere to the "three-scratch test" as described by us formerly, *i. e.* three short parallel scratches on the animal, just deep enough to draw blood in about half a minute.

It is evident that the cutaneous and intracutaneous methods, however modified and improved they may be, depend essentially on the same principles. Every worker obtains the best results with the method which he has himself perfected, and it is hard for him to realise that had he applied his skill to the perfection of another test he might now prefer that to his own.

We still believe, however, that our preference for the intracutaneous method rests not so much on the personal factor as on the relative merits of the two tests:

(1) Whereas in the intracutaneous test we are using dilutions of 1/1000–1/8000, in the von Pirquet test the dilutions range from 1/5–1/40.

(2) The exact amount of material absorbed by any scratch cannot be determined.

(3) The material applied to a scratch can be wiped or licked off.

(4) The "scratch" is not only more irritant at the time of the test, so that the animals may obscure the reactions by extraneous scratching, but the resulting wound heals more slowly than the intracutaneous lesion.

(5) The difficulty of standardising the von Pirquet method is obvious.

Even if any worker succeeds in regulating the length and depth of the scratches, the variations in thickness of the skin have to be taken into account, and also differences in the method of application of the tuberculin dilutions.

The technique of intracutaneous injection, though more difficult to acquire, is assuredly more suitable for a standard reaction.

THE STANDARD TUBERCULIN.

This is a preparation of undiluted bovine tuberculin. The original standard (S1)* was chosen towards the close of 1919; it was used throughout 1920 and during part of 1921. In the later year its place was taken by a second preparation (S2), which has been in use since. The second standard is as far as possible identical in composition with the first, and is equal to the first in strength as judged by von Pirquet intracutaneous and subcutaneous tests. The latter are shown in Tables IV and V.

The former shows comparative tests on these two preparations at different times. Four different batches of animals were used, one of which was of standard sensitivity (11/11/20), the second (29/9/20) was supersensitive, while the third and fourth were subsensitive. But the two standards are of equal strength as judged by these tests, which confirm the results given by the intracutaneous method.

Table V shows the specific toxicity of S1 and S2 during the time that they have been used as standards.

In this table are included all animals injected with S1 and S2 in the main subcutaneous test. Thus all the animals were sensitive, some were supersensitive, and a few of less than standard sensitivity.

It will be seen that at 0·25 c.c. the death-rate is practically 100 per cent., at 0·1 c.c. practically 50 per cent., and that these figures are reasonably consistent.

The death-rate at 0·05 c.c. and 0·04 c.c. may at first sight appear anomalous, but it must be remembered that only in very sensitive batches are many animals injected with these doses, and that where many animals die at 0·05 c.c. few or none need be injected with 0·1 c.c. The higher doses, 1 c.c. and 0·5 c.c., on the contrary, are only used when the batch is subsensitive, and in such cases we shall find survivals at the 0·25 c.c. level (Table V).

Of the 261 animals injected we see that 69 received 0·25 c.c., and of this number 66 died, *i. e.* 96 per cent.; 114 received 0·1 c.c., and of these 50 died, *i. e.* 43·9 per cent.

The variations from year to year cannot be considered as due to the standard tuberculin but to the sensitivity of the animals, and this for two reasons:

(1) This last year gives as high a death-rate at 0·25 and 0·1 c.c. doses as any preceding year. If variations were due to the tuberculin we should expect a loss of toxicity and not a gain.

(2) Comparisons of the same standards S1 and S2 with other samples (dried and liquid) at different times during the last three years give consistent results.

We may mention that a sample of Koch's tuberculin made many years before the war was found quite recently by us to be equal in strength to our standard and to tuberculin made by us in 1922 from the strain used originally by Koch.

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* This was equal in strength to Ehrlich's standard (*vide supra*).

Owing to the slight differences in sensitivity of batches of animals, even when all precautions are taken, we are unable to give an absolute minimal reacting dose or lethal dose for the standard. We can only say that with a batch of standard sensitivity, the standard tuberculin will give a strong reaction at 1/4000 by the intracutaneous test, at 1/20 and possibly 1/40 by the von Pirquet test, and will kill 100 % of animals at 0·25 c.c. and 50 % at 0·1 c.c.

Samples of unknown strength must be compared with the standard tuberculin, which is the only absolute constant in the test.

With regard to dried preparations, we have tested three since September, 1921. These were all made by precipitation from old tuberculin. They all three appear so far to be stable as judged by periodic comparisons with the liquid standard. In view of the stability of the latter and its convenience, there seems to be little profit in substituting a dry for a wet standard. Moreover the expense would be very considerable compared with the advantage (if any) gained. We are keeping these dried preparations, but at present do not intend to substitute them for S2.

The most interesting thing about them is that although they were all made in the same way, except for details in methods of precipitation, they differed very much in strength, 5 grm. of DT1, 20 grm. of DT2 and 50 grm. of DT3 being required to make 100 c.c. of standard tuberculin respectively (*vide* Table VI). This result seems to have an important bearing on the use of various ophthalmic tuberculins for veterinary and human use. These are wet or dry preparations, many of them made by precipitation of old tuberculin (T or PT) by alcohol. Unless they are standardised, their use is fraught with chance of error.

Our standard for human tuberculin (T) is the same as for bovine tuberculin (PT). Our standard for T.O.A. and for P.T.O. is one-tenth of this.

The ideal standard fulfils the following conditions:

(1) It is stable.

(2) It is reasonably attainable.

(3) It is a true measure of clinical value.

With regard to stability, we have shown that so far as we can judge our standard tuberculin is stable when kept undiluted. Moreover, tuberculins of the same strength can be produced year after year in the laboratory. A comparison of our standard with Frankfort tuberculin of different years is shown in Table VII.

In these laboratories our present standard was adopted for P.T. at the end of 1919. Since then many litres of undiluted tuberculin have been prepared, and all of this has been up to standard strength. The same standard was adopted for human tuberculin in August, 1921, and since then, of the total amount prepared only about 6 % has failed to pass the test.

The bovine culture which we use constantly for the preparation of tuberculin sent by us to another laboratory which had for some time been having trouble with the manufacture of tuberculin. Table VIII shows the test of tuberculin made in that laboratory before and after we sent them our culture

"B 4." We have ourselves experienced a period during which we could not obtain tuberculin from human strains. We found that our cultures of human bacilli, although filming well, yielded only a minute amount of tuberculin as judged by animal test. When we came to test the cultures by injection into rabbits and guinea-pigs we found that they were avirulent. They had become so in process of time, and the exact date of the change could not be determined.* We know, however, that at some period before 1915 they had yielded good tuberculin. Fortunately all strains of tubercle bacilli do not seem to undergo this change. Our best bovine strain has been maintained in artificial culture for at least ten years and still yields tuberculin of standard strength. Koch's original human strain received by us in 1921 yielded tuberculin equal to our standard as soon as we could obtain filming cultures. The possibility of such a change must, however, be borne in mind, and renders imperative constant routine standardisation of all the tuberculin produced.

We may say, then, that given a suitable strain of human or bovine tubercle bacilli, tuberculin can be easily obtained of a strength equal to our standard preparation. We have ourselves used four different strains for tuberculin production during the last two years and all of them were suitable.

In a recent series of tests on tuberculin from various sources two-thirds of the samples were found to be of standard strength. Tests on various samples from England, the Continent and America are shown in Table IX. Most of them were of standard strength. It may be fairly claimed that we have not set our standard too high but that it is reasonably attainable.

The standard satisfies two of the three criteria. It is stable and attainable. Is it a true estimate of clinical value? That is to say, if a tuberculin passes the standard test, is it sufficiently potent for clinical (human and veterinary) use and is it specific? We have no means at this laboratory of testing these points, but we have accumulated records of human and veterinary diagnostic work which are sufficient to convince us that the standard adopted is suitable in these respects. Moreover, as it is founded on the Ehrlich standard and equal in strength to the official Frankfort standard, all the evidence adduced in favour of these preparations may be claimed as its heritage.

Whatever improvement be introduced hereafter, the tests must always depend on the same principle; there will always be the variable factor of specific sensitivity; there will always be the need for a well-tried standard. This we claim to have provided; nor will the inevitable modifications of the future alter the value of the standard which was determined in the past.

In conclusion we have to thank Dr. R. A. O'Brien for the interest he has always taken in the development of this work, which he himself initiated, and our colleague Dr. C. C. Okell for active assistance throughout the last two years.

SUMMARY.

A description is given of the method used in these laboratories for the standardisation of tuberculin.

While it is inevitable that the test will be modified in detail and improved as time goes on, its fundamental principles must remain unchanged.

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* The possibility of an error in labelling cannot be absolutely excluded.

This being so, a standard tuberculin is essential, which must be stable, reasonably attainable, and a true laboratory measure of field and clinical potency.

Our standard, based on Ehrlich's original standard, fulfils these conditions.

REFERENCES.

Eagleton, A. J.—(1921) *Lancet*, **1**, 429.

Koch, R.—(1891) *Deutsch. med. Wchnschr.*, **43**, 1189.

Loewenstein, E.—(1913) "Anwendung des Tuberculins beim Menschen," *Handbuch der pathogenen Mikroorganismen* (Kolle and Wassermann), **5**, 549.

Otto, R.—(1904) *Klin. Jahrb.*, **13**, 1.

Otto, R., and Hetsch, H.—(1921) "Die staatliche Prüfung der Heilsera und des Tuberkulins," *Arbeiten aus dem Staatsinstitut für experimentelle Therapie und dem Georg Speyer Haus zu Frankfurt a. M.*, Heft. 13, p. 107.

Reeser, H. E.—(1908) *Cntrlbl. f. Bakt.*, Orig., **46**, 166.

Schroeder, E. C., and Brett.—(1919) *J. Amer. Vet. Med. Assoc.*, **4**, 357.

Table I.—*Standardisation of Tuberculin.*

60 guinea-pigs injected with living T.B. (Emulsion from "glycerin" Dorset or rabbit lung.)

A. After ten days. Preliminary intracutaneous test.
Standard 0·2 c.c. diluted 1/500, 1/1000, 1/2000.

B. Main intracutaneous test (when intracutaneous test 1/2000 = + +*).

	1/1000.	1/2000.	1/4000.	1/8000.
Standard	+ +	+ +	± ±	±
Sample "Q"	+ +	+ +	± ±	$\underline{\pm}$

C. Preliminary subcutaneous test (when intracutaneous test = + +, and with careful selection of guinea-pigs).

Standard 0·25 c.c. = s.
(One week's interval.)
,, 0·25 c.c. = †.
,, 0·1 c.c. = †.
,, 0·1 c.c. = s or x.

D. Main subcutaneous test (when preliminary test complete).

Standard 0·1 c.c. = †.
,, 0·1 c.c. = s.
Sample "Q" 0·1 c.c. = †.
,, 0·1 c.c. = s.

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* The following abbreviations are used in all tables: † = typical death; s = survival; ss = seedy but survived; x = atypical death; + +, ± ±, +, ±, $\underline{\pm}$, − = degrees of reaction; n = no test.

TABLE II.—*Value of Using Survivors from Subcutaneous Test.*

Date.	Dose.	Standard.	Sample A.
30 . 6 . 20	0·5 c.c.	†	s
	0·25 c.c.	†	s
	0·1 c.c.	†	s
1 . 7 . 20	0·1 c.c.	†	n
	0·05 c.c.	ss	n
	0·5 c.c.	n	s
8 . 7 . 20	2·0 c.c. (guinea-pig A)	n	s
12 . 7 . 20	1·5 c.c. (,, B)	n	s
	0·1 c.c. (,, A)	†	
(same animals as on 8 . 7 . 20)	0·1 c.c. (,, B)	†	

TABLE III.

	Dose.	Standard.	Sample A.	Sample B.	Sample C.	Sample D.	Sample E.	Sample F
Batch 63—								
24 . 5 . 23	0·25 c.c.	†	—	—	—	—	—	—
	0·1 c.c.	† †	† † †	† s	†	—	†	—
	0·05 c.c.	s s	s s s	s	†	—	s	—
Batch 64—								
25 . 5 . 23	0·25 c.c.	†	—	—	—	—	—	—
	0·1 c.c.	† ss	s s	s s x	—	—	—	† s
	0·05 c.c.	s	—	—	† s	—	—	—
Survivors from Batches 63 and 64—								
1 . 6 . 23	0·25 c.c.	†	ss	†	†	†	—	—
	0·1 c.c.	† s	ss s	† s	s s	† s	—	—
	0·05 c.c.	s	s	s	—	s	—	—

Samples A, B, D, E and F were, on the intracutaneous test, equal to the standard.

TABLE IV.—*Subcutaneous Tests in Parallel on Standards S1 and S2.*

Dose.	11 . 11 . 20.		29 . 11 . 20.		9 . 2 . 21.		19 . 2 . 23.	
	S1.	S2.	S1.	S2.	S1.	S2.	S1.	S2.
0·5 c.c.	—	—	—	—	—	—	†	†
0·25 c.c.	†	†	—	—	†	†	† †	† †
0·1 c.c.	ss	†	—	—	s	s	s s	s s
0·05 c.c.	s	s	—	—	—	—	s	s
0·04 c.c.	n	n	† †	† x	—	—	—	—
	—	—	s s	s s	—	—	—	—

TABLE V.—*Subcutaneous Tests on Two Standards* 1919–1923 (261 *animals used*).

Dose.	S1, 1919.			S1, 1920.			S2, 1921.			S2, 1922.			S2, 1923.		
	†	Total injec.	% †	†	Total injec.	% †	†	Total injec.	% †	†	Total injec.	% †	†	Total injec.	% †
1·0 c.c.	1	1	100	—	—	—	—	—	—	—	—	—	—	—	—
0·5 c.c.	12	12	100	—	—	—	—	—	—	1	1	100	1	1	100
0·25 c.c.	17	18	94	4	4	100	24	25	92	15	15	100	6	6	100
0·15 c.c.	—	—	—	—	—	—	3	4	75	—	—	—	—	—	—
0·1 c.c.	11	20	55	6	11	54	7	18	40	13	30	43	9	17	53
0·05 c.c.	5	8	62·5	7	15	47	7	18	40	4	11	36	0	5	0
0·04 c.c.	—	—	—	2	4	50	2	4	50	—	—	—	—	—	—
0·025 c.c.	0	6	0	0	1	0	0	2	0	0	3	0	—	—	—

TABLE VI.—*Comparison of Three Dried Tuberculins—DT1, DT2, DT3 with S2.*

By intracutaneous tests DT1 (5%) = S2 Sept., 1921, and Feb., 1922.
,, ,, ,, DT2 (20%) = S2 May, 1922, and Jan., 1923.
,, ,, ,, DT3 (50%) = S2 Jan., 1923.

Subcutaneous Test.

Dose.	Sept., 1923.		May, 1922.		Feb., 1923.	
	DT1 5%.	S2.	DT2 20%.	S2.	DT3 50%.	S2.
0·5 c.c.	—	—	†	—	†	†
0·25 c.c.	—	—	†	†	† s	† †
0·1 c.c.	†	†	s	†	s ss	ss s
0·05 c.c.	† † s s	† †	—	—	—	—
0·04 c.c.	† s	s	—	—	—	—
0·025 c.c.	s s	s	—	—	—	—

TABLE VII.—*Comparison of S1 and S2 with Ehrlich Original Tuberculin* (= *Eh.*) *and Frankfort Standard of* 1921 (= *Fr.*).

Date.	Dose.	Subcutaneous test.			Result of intracutaneous test.
		S2.	Eh.	Fr.	
23 . 9 . 21	—	—	—	—	S1 = Eh.
17 . 10 . 21	0·25 c.c.	†	†	—	—
	0·1 c.c.	†	ss	—	—
	0·05 c.c.	ss	†	—	—
1 . 11 . 21	—	—	—	—	S2 = Eh.
18 . 1 . 22	0·25 c.c.	†	—	—	—
	0·1 c.c.	† s	† s	—	—
	0·05 c.c.	—	s s	—	—
24 . 1 . 22	—	—	—	—	Fr. = S2 (also von Pirquet Fr. = S2)
1 . 2 . 22	0·2 c.c.	†	—	†	—
	0·1 c.c.	—	—	† †	—
	0·05 c.c.	—	—	s	—

TABLE VII—*continued.*

Date.	Dose.	Subcutaneous test.			Result of intracutaneous test.
		S2.	Eh.	Fr.	
6 . 2 . 23	—	—	—	—	Fr. = S2
14 . 3 . 23	—	—	—	—	Fr. = S2
24 . 5 . 23	0·25 c.c.	†	—	—	—
	0·1 c.c.	† †	—	† s	—
	0·05 c.c.	s s	—	s	—
25 . 5 . 23	0·25 c.c.	†	—	—	—
	0·1 c.c.	† ss	—	s s x	—
	0·05 c.c.	s	—	—	—
1 . 6 . 23	0·25 c.c.	†	—	†	—
	0·1 c.c.	† s	—	† s	—
	0·05 c.c.	s	—	s	—

TABLE VIII.—*Tuberculin from Laboratory A.*

Date.	Dose.	S1.	S2.	Laboratory A.
27 . 10 . 19	0·5 c.c.	†	—	s
	0·25 c.c.	†	—	s
	0·1 c.c.	s	—	s
30 . 6 . 20	0·5 c.c.	†	—	s
	0·25 c.c.	†	—	s
	0·1 c.c.	†	—	s
1 . 7 . 20	0·5 c.c.	—	—	s
	0·1 c.c.	†	—	—
	0·05 c.c.	s	—	—
8 . 7 . 20	1·5 c.c.	—	—	s
	2·0 c.c.	—	—	s
25 . 5 . 23	0·25 c.c.	—	†	By intracutaneous test = S2 (after receiving B4)
	0·1 c.c.	—	† ss	s s
	0·05 c.c.	—	ss	—
1 . 6 . 23	0·25 c.c.	—	†	†
	0·1 c.c.	—	† s	† s
	0·05 c.c.	—	s	—

Note apparent discrepancy between intracutaneous and subcutaneous tests 25 . 5 . 23.

TABLE IXA.—*Comparison against Standards of Two Commercial "Bovine" Tuberculins, N1 and N2, issued from a Laboratory in Different Years.*

Date.	Dose.	Subcutaneous test.				Other tests.
		S1.	S2.	N1.	N2.	
23 . 11 . 20	0·5 c.c.	—	—	†	—	By von Pirquet N1 = ½ S1
	0·25 c.c.	†	—	† †	—	—
	0·1 c.c.	†	—	s	—	By intracutaneous N1 = ½ S2
	0·05 c.c.	† †	—	s	—	—
	0·025 c.c.	ss	—	—	—	—
24 . 5 . 23	—	—	—	—	—	By intracutaneous N2 = S2
25 . 5 . 23	0·25 c.c.	—	†	—	—	—
	0·1 c.c.	—	† ss	—	† †	—
	0·05 c.c.	—	s	—	—	—

Note.—N1 is only half strength; N2 = standard.

Table IXb.—*Comparison against Standards of Tuberculins P1, diluted for Veterinary Use, and P2, undiluted.*

Date.	Dose.	S1.	S2.	P1.	P2.
6 . 12 . 20	2·0 c.c.	—	—	—	—
	1·0 c.c.	—	—	†	—
	0·75 c.c.	—	—	† †	—
	0·5 c.c.	—	—	s s	—
	0·1 c.c.	†	—	—	—
	0·05 c.c.	† † † x s	—	—	—
	0·025 c.c.	s	—	—	—
25 . 5 . 23	0·25 c.c.	—	—	—	—
	0·1 c.c.	—	† ss	—	† s

Note.—P1 is only 1/15 instead of 1/10 strength of standard, while P2 = standard.

Table IXc.—*Tests on Veterinary Tuberculin from Laboratory D.*

Date.	Dose.	S2.	D1.
6 . 2 . 23	—	—	By intracutaneous test $= \frac{S}{20}$
25 . 5 . 23	0·25 c.c.	†	—
	0·1 c.c.	† ss	—
	0·05 c.c.	s	—
	2·0 c.c.	—	†
	1·0 c.c.	—	s

Note.—D1 is only 1/20 standard instead of 1/10.

Table IXd.—*Tests on Tuberculins from Laboratory R, R1 (diluted for Veterinary Use) and R2 (undiluted).*

Date.	Dose.	S2.	R1.	R2.
24 . 5 . 23	—	—	By intracut. test $= \frac{S}{10}$	—
18 . 4 . 23	—	—	—	By intracut. test = S
3 . 5 . 23	0·25 c.c.	†	—	—
	0·1 c.c.	† x † s	—	† s

Table IXe.—*Tests on Tuberculins from Laboratory A.*

Date.	Dose.	S2.	A1.	A2.	A3.	A4.
29 . 5 . 22	—	—	By intracut. test = S2	—	—	—
	0·2 c.c.	†	†	—	—	—
	0·1 c.c.	ss	†	—	—	—
4 . 7 . 22	0·1 c.c.	†·†	s*	—	—	—
	0·05 c.c.	† s	† †	—	—	—
	0·025 c.c.	—	s	—	—	—
3 . 23	0·25 c.c.	†	—	By intracut. test = S2	—	—
	0·1 c.c.	† †	—	†	s s	† s
	0·05 c.c.	s s	—	†	—	—

Note.—A1 and A4 = standard, A2 is stronger, A3 weaker.

* *Note.*—Survival out of order; animal not sensitive.

Table IXf.—*Tests on Various Tuberculins from Laboratory B.*

Date.	Dose.	S2.	B1.	B2.	B3.	B4.	B5.
10.11.21	—	—	By von Pirquet test = S2	—	—	—	—
24.1.22	—	—	By von Pirquet test = S2	—	—	—	—
29.5.22	—	—	By von Pirquet test = S2	—	—	—	—
31.1.22	0·2 c.c.	†	†	—	—	—	—
	0·1 c.c.	ss	†	—	—	—	—
	0·1 c.c.	† †	†	—	—	—	—
	0·05 c.c.	† ss	† ss	—	—	—	—
	0·015 c.c.	—	s	—	—	—	—
3.23	0·25 c.c.	†	—	By intracut. test = >S2	—	—	—
24.5.23	0·1 c.c.	† †	—	† †	—	—	—
	0·05 c.c.	s s	—	s s	—	—	—
25.5.23	0·25 c.c.	†	—	—	—	—	—
	0·1 c.c.	† ss	—	—	s s	s s	†
	0·05 c.c.	s	—	—	—	—	s

Table IXg.—*Tests on Tuberculins from Laboratory C, prepared in successive years.*

Date.	Dose.	S2.	C1922.	C1923.
28.6.22	—	—	By intracut. test = S2	—
4.7.22	0·1 c.c.	†	†	—
	0·05 c.c.	ss	s	—
10.4.23	—	—	By intracut. test = S2	—
25.5.23	0·25 c.c.	†	—	—
	0·1 c.c.	† ss	—	† s
	0·05 c.c.	s	—	—

THE PLATELET-COUNT IN RATS SUFFERING FROM VITAMIN-A DEFICIENCY.

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In a recent communication on this subject by Cramer, Drew and Mottram (1923), an attempt has been made to explain the divergence between their findings and ours with respect to the platelet content of the blood of rats suffering from a vitamin A deficiency. Briefly stated the position is as follows: Cramer, Drew and Mottram (1922) produced evidence to show that the deprivation of young growing rats of the fat-soluble vitamin resulted in a severe thrombopœnia. This diminution in the platelet-count—from 1,000,000 to 300,000 or even less per c.c.—was found by them to be such a constant and characteristic feature of vitamin A deficiency in the rat that they termed it the specific lesion of this condition, and based on this observation an explanation of the diminished resistance to infection shown by rats suffering from such a deficiency. With a view to employing this reduction in platelets as a measure of the degree of vitamin A deficiency we repeated this work, and obtained results which were not in keeping with the findings of Cramer, Drew and Mottram (Bedson and Zilva, 1923). In our experience the withholding of vitamin A from the diet of growing rats does not result in a severe thrombopœnia. Counts made on rats in the last stages of this deficiency showed a reduction which in many cases was so small as to be within the experimental error of the technique employed, and in the most marked cases did not represent more than a 20 per cent. reduction. It is now claimed by Cramer, Drew and Mottram (1923) that the optical system employed by us for the counting of platelets was defective, and that owing to this the true nature of certain bodies or "protoplasmic *débris*," which, according to these workers, appear in the blood of vitamin A deficient rats, has been overlooked, leading to their inclusion by us in the platelet-count. They produce figures in support of this contention. (The claim that bacteria or lipoid globules could be mistaken for platelets is not worthy of consideration.) Objection is taken to the employment of an Abbé condenser in our optical system, which, as they correctly point out, reduces the resolving power of the objective; in their system the condenser is of the oil-immersion type with a working N.A. of 1·4. We were fully aware of the defects of the Abbé condenser before reading their communication, but surely such refinements in optical technique are hardly necessary when dealing with objects, the average diameter of which is in the neighbourhood of 3μ. However, the issue is a perfectly straight one: Does the blood of a vitamin A deficient rat contain these protoplasmic *débris*—what one might term pseudo-platelets? If so are they present in such numbers as to explain the divergence in findings already referred to? It is hardly necessary to say that before publishing our findings, to which Cramer, Drew and Mottram take exception, this point had engaged our attention. A careful morphological study of the blood of deficient rats was made in the hope of finding an explanation of the differences in findings. None was forthcoming. These observations have been repeated in greater detail and form the subject of this communication.

PARALLEL PLATELET COUNTS MADE WITH THE TWO OPTICAL SYSTEMS.

The blood of normal and deficient rats was collected from the tail by the technique described by Cramer. The diluent employed was 2 per cent. sodium citrate in normal saline. Drops of this citrated blood were mounted between slide and coverslip, ringed with vaseline, and each preparation counted with the two optical systems. These two optical systems had the following composition:

TABLE I.

System.	Objective.	Ocular.	Condenser.
A.	Leitz 2 mm. apochromat. N.A. 1·4	Koristka 8, compensating	Zeiss oil-immersion, N.A. 1·4.
B.	Koristka 1/15″ semi-apochromat. N.A. 1·3	„	Abbé.

At the same time drops of the citrated blood were spread on slides as ordinary blood smears, fixed in methyl alcohol and stained by Giemsa.

These stained preparations were examined for the presence of abnormal bodies similar in size to platelets, and in two instances the red cell : platelet ratio was estimated as well.

The results are tabulated below:

TABLE II.

Animal.	Red cell : platelet ratio.			Remarks.
	Op. system A.	Op. system B.	Stained smear.	
Normal rat	8·4	8·6	—	—
—A rat 3848	10·4	10·9	10·9; no abnormal forms seen	Well-marked eye change in both eyes.
—A rat 3713	11·3	12·0	No abnormal forms seen	Well-marked eye change.
—A rat 3858	9·8	9·4	9·7; no abnormal forms seen	Commencing eye change in both eyes.

It will be seen that the counts obtained with the two optical systems show little or no difference, and the fact that the elements counted by us in the unstained preparations were really platelets is borne out by the counts made on the fixed and stained blood, where no doubt can exist as to what is and what is not a platelet.

COMPARISON OF DILUTING FLUIDS.

As the diluent used by Cramer, Drew and Mottram was Toisson's fluid, it was necessary to see whether the blood of deficient rats collected in this diluent gave the same counts as those obtained with the citrate-saline solution which we employ. The results of this experiment given in Table III show that the two diluents are equally good.

TABLE III.

Animal.	Red cell : platelet ratio.		Remarks.
	Toisson's fluid.	2% citrate in saline.	
—A rat 3859	9·7	10·4	No eye change. Weight stationary for 16 days.

MORPHOLOGICAL STUDY OF THE BLOOD OF DEFICIENT RATS.

In view of the findings given above it seemed hardly necessary to proceed further with this inquiry. However, this morphological examination was carried out anew on the blood of the deficient rats under investigation. Blood smears were made in the usual manner, and smears were prepared from citrated blood collected from the tail as for platelets counts. The diluent employed was either the ordinary citrate-saline, or with the addition of a fixative such as formalin (0·5 per cent.) or osmic acid (0·1 per cent.). The addition of a fixative to the diluting fluid is an advantage, and has been made use of by us in counting platelets for some time. The blood elements are fixed as they enter the anti-coagulant and their preservation is therefore ensured. Formalin has this defect, however, as a platelet fixative—it does not preserve the granules well. In this respect osmic acid is vastly superior. These preparations were stained either by Giemsa after fixation with methyl alcohol, or by the supra-vital method of Sabrazes (1923). This latter method consists in placing a drop of carbol-toluidine blue on the dried but unfixed smear, covering with a cover-slip and examining immediately. The smear should be freshly made. By this method the platelets stain a bluish-grey, their granules taking on a deep purple-black colour. As pointed out by Sabrazes, this is undoubtedly the simplest and most efficient method for the demonstration of the platelet granule, and incidentally the azurophil granules of the lymphocyte and large mononuclear leucocyte. The result of this morphological study of the blood of vitamin A deficient rats simply went to confirm our previous findings. No evidence whatever was obtained of the occurrence of protoplasmic fragments in this condition, the only elements observed being the red cells, leucocytes and platelets.

THE EFFECT OF ANTI-PLATELET SERUM IN DEFICIENT RATS.

In order to make this investigation as complete as possible it was decided to examine the effect of anti-platelet serum on the blood of vitamin A deficient rats. This serum contains an antibody specific for platelets, and it has been noted that its dosage can be so graded that the effect *in vivo* is limited to a reduction in platelets amounting to almost complete disappearance from the circulation, without the production of hæmorrhages and therefore without any disturbance in the red count. An anti-rat platelet serum was prepared in rabbits with platelets which were free from red cell contamination on microscopical examination. This serum was titrated in normal rats of from 70–80 grm. weight, and was found to produce almost complete disappearance of platelets from the circulation in 24 hours when inoculated intraperitoneally in a dose of 0·15 c.c. No purpura resulted from such a dose with the exception of an occasional skin hæmorrhage. Two rats suffering from a vitamin A deficiency were inoculated intraperitoneally with the above dose of anti-platelet serum, and the changes noted in the blood are tabulated below:

TABLE IV.

Animal.	Time of observation.	Weight.	Red cells.	R/P.	Platelets.	Leucocytes.	P.M. findings.
—A rat 3985	Before inoculation	69 gms.	8,280,000	8·5	975,000	10,660	—
	7.8.23 2.30 P.M.	0·15 c.c. anti-platelet serum inoculated intra-peritoneally.					
	8.8.23 10.30 A.M.	—	8,320,000	151·7	54,000	20,200	Some small petechiæ in abdominal wall at site of inoculation. One skin hæmorrhage seen. Otherwise normal.
—A rat 3989	Before inoculation	76 gms.	7,440,000	10·0	744,000	8,400	—
	7.8.23 2.30 P.M.	0·15 c.c. anti-platelet serum inoculated intra-peritoneally.					
	8.8.23 11.30 A.M.	—	7,080,000	356·3	20,000	20,200	No hæmorrhages in skin or subcutaneous tissue. Peritoneal cavity contains a small quantity of blood-stained fluid. Otherwise normal.

It will be seen that in these two rats, as in normal rats of approximately the same weight, the result of the inoculation has been the almost complete disappearance of platelets from the circulation. The red cells are unaltered in number, whilst the leucocytes have undergone a definite increase in both cases. If the blood of these rats had contained "pseudo-platelets" one would have expected these elements to have remained unaffected, only the true platelets disappearing from the circulation. This was not the case; those elements counted by us as platelets had almost entirely disappeared. Then, again, if the true platelet content of the blood of these deficient animals had been only 200,000 or 300,000, one would have expected that this dose of serum would have produced a greater effect in them than it did in the normal rats. The degree of purpura was precisely similar in both categories of animals, *i. e.* none at all, or else a rare skin hæmorrhage.

DISCUSSION.

From the above findings there can be no doubt that the elements counted by us in deficient rats were in reality platelets, and that the difference between the optical system used by us and the one employed by Cramer, Drew and Mottram was not responsible for the divergence of findings. This is what one would have expected, for their claim did not really bear a close scrutiny. An analysis of their series of parallel counts, made on the blood of deficient rats with the two optical systems, reveals the fact that as the number of platelets decreases the protoplasmic fragments increase in number, the sum of the two remaining more or less constant. This alone should have made them suspect their explanation. Then, again, no mention is made in their communication of any examination of stained preparations of the blood of deficient rats. Had these authors seen these "protoplasmic *débris*" in fixed and stained preparations? What were their staining reactions? What form did these fragments present in fixed preparations? In the absence of any data on these points one is forced to conclude that these workers based their claim chiefly, if not entirely, on the examination of the unstained fresh blood only. Surely this was rather slender evidence from which to draw their conclusions, and hardly warrants the attack which they made on our technique! What, then, is the explanation of this discrepancy in findings? We would make the following suggestions: Contrary to the opinion expressed by Cramer, Drew and Mottram we find that the deficient rat bleeds with difficulty, and this becomes more and more marked as the deficiency becomes more pronounced; in fact, in some instances we have been unable to procure a reliable preparation from the tail, and have had to resort to cardiac puncture. It is an axiom in platelet counting that the blood must flow readily into the anticoagulant, otherwise platelets are lost or else partially altered by commencing coagulation and distorted in shape. Is it not possible that the "protoplasmic *débris*" of Cramer, Drew and Mottram are nothing more than altered and distorted platelets?

CONCLUSIONS.

(1) The optical system employed by us for the counting of platelets was not defective inasmuch as it was capable of performing the work it was called upon to do.

(2) We have been unable to confirm the observation of Cramer, Drew and Mottram as to the presence of "protoplasmic *débris*" in the blood of rats suffering from a vitamin A deficiency.

(3) The elements counted by us as platelets in the blood of such rats were in reality true platelets.

(4) We repeat that we are unable to confirm the original observation of Cramer, Drew and Mottram that vitamin A deficiency in the rat is productive of a severe thrombopœnia.

REFERENCES.

Bedson, S. P., and Zilva, S. S.—(1923) *Brit. J. Exper. Pathol.*, **4**, 5.
Cramer, W., Drew, A. H., and Mottram, J. C.—(1923) *Ibid.*, **4**, 37.
Idem.—(1922) *Proc. R. Soc.*, B, **93**, 449.
Sabrazès, J.—(1923) *Compt. rend. Soc. de Biol.*, **88**, 697.

THE INFLUENCE OF INSULIN ON THE FORMATION OF GLYCOGEN.

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An attempt was made, in the following experiments, to solve the question of the fate of the sugar disappearing from the blood after administration of insulin.

METHODS.

The experiments were performed on rabbits, the usual food of which consisted of cabbage and oats; in some cases the glycogen in the tissues was increased by a diet of carrots, sugar and oats. In one series of experiments, in order to get the liver and muscles free from glycogen, hypodermic injections of adrenaline were given the day before (Drummond and Paton, 1904; Agadschanianz, 1907). The animal received, per kilo weight, 1 c.c. of 1 : 1000 adrenaline solution (Parke, Davis & Co.), diluted to 2 c.c. with distilled water. In the earlier experiments I injected larger doses—from 1·5 to 2 c.c. adrenaline per kilo.

The blood-sugar was determined by Maclean's (1919) 1 c.c. method. The glycogen was estimated by Pflüger's method in 10 gm. of liver (pieces of liver were taken from different parts of the organ), in 50 gm. skeletal muscles (different muscles of hind legs), and in the whole heart. The sugar, obtained by hydrolysis of the glycogen, was estimated by Bertrand's method.

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* The results of this investigation have been reported in a meeting of the Physiological Society, May 19th, 1923.

GLYCOGEN CONTENT OF THE LIVER AND MUSCLES AFTER ADMINISTRATION OF INSULIN.

Macleod and his co-workers have observed in depancreatised dogs under the influence of insulin, a storage of glycogen in the liver (Banting *et al.*, 1922). I have repeated these experiments, but on normal rabbits.

From Table I we see that the glycogen content of the liver and muscles in very well nourished rabbits (fed on carrots, sugar and oats) after administration of insulin is markedly lowered as compared with animals fed in the same way but without injection of insulin. In no cases have convulsions been observed, although the sugar content of the blood fell distinctly.

TABLE I. INFLUENCE OF INSULIN ON THE GLYCOGEN CONTENT OF LIVER AND SKELETAL MUSCLES

RABBIT	DATE	DIET	WEIGHT IN gm	INSULIN INJECTED	PER CENT. OF INITIAL BLOOD-SUGAR	LOWEST PER CENT. OF BLOOD-SUGAR	PER CENT OF GLYCOGEN	
							LIVER	MUSCLES
II	28th FEB	CARROTS & SUGAR AFTER ADRENALINE	1822	—	—	—	4·34	0·44
XII	13th APRIL	CARROTS SUGAR AND OATS	2920	—	—	—	11·12	0·85
VI	15th MARCH	CARROTS AND SUGAR	1835	0·02 gm CRUDE INSULIN	0·112	0·02 IN 2 hrs 45 mins	1·90	0·14
XVI	14th MAY	CARROTS, SUGAR AND OATS	3168	0·007 gm INSULIN HYDROCHLORIDE	0·135	0·03 IN 2 hrs	2·87	0·09

NOTE. RABBIT II ON THE 26th FEB. RECEIVED AT 3 P.M. AN INJECTION OF 1·5 CC. OF ADRENALIN + 1 5 CC OF DISTILLED WATER, AT 6 P.M. FOLLOWED THE SECOND INJECTION OF 1·0 CC OF ADRENALIN + 1 0 CC OF WATER ON 27th FEB. FROM 12 A.M FEEDING WITH CARROTS AND SUGAR, KILLED ON 28th FEB. AT 12 15 PM.. RABBIT VI WAS KILLED 4½ HOURS AFTER ADMINISTRATION OF INSULIN; RABBIT XVI 5 HOURS AFTER. THESE TWO RABBITS AND RABBIT XII WERE NOT TREATED WITH ADRENALIN BEFORE FEEDING WITH CARROTS AND SUGAR.

The disappearance of glycogen from both liver and skeletal muscles in animals which are given a dose of insulin sufficient to cause hypoglycæmic convulsions was observed independently by Dudley and Marrian (1923).

INFLUENCE OF INSULIN IN CASES OF ARTIFICIAL GLYCOSURIA.

I tried to obtain in rabbits a very high artificial hyperglycæmia, and to define (*a*) the influence of insulin on it, and (*b*) the influence of insulin on the storage of glycogen.

Influence of Insulin on the Blood-sugar.

In fasting rabbits of 2–3 kilos injected the day before with adrenaline, I introduced subcutaneously in control experiments large doses (20–30 gm.) of glucose. The concentration of the solution employed varied from 10 to 13 per cent. It was injected during the first 2–3 hours, the experiment lasting 4–5 hours.

In the main experiments the procedure was the same except that glucose and insulin were injected subcutaneously also in large doses (from 40 to 60 mgm. crude insulin, 10 to 20 mgm. per injection).

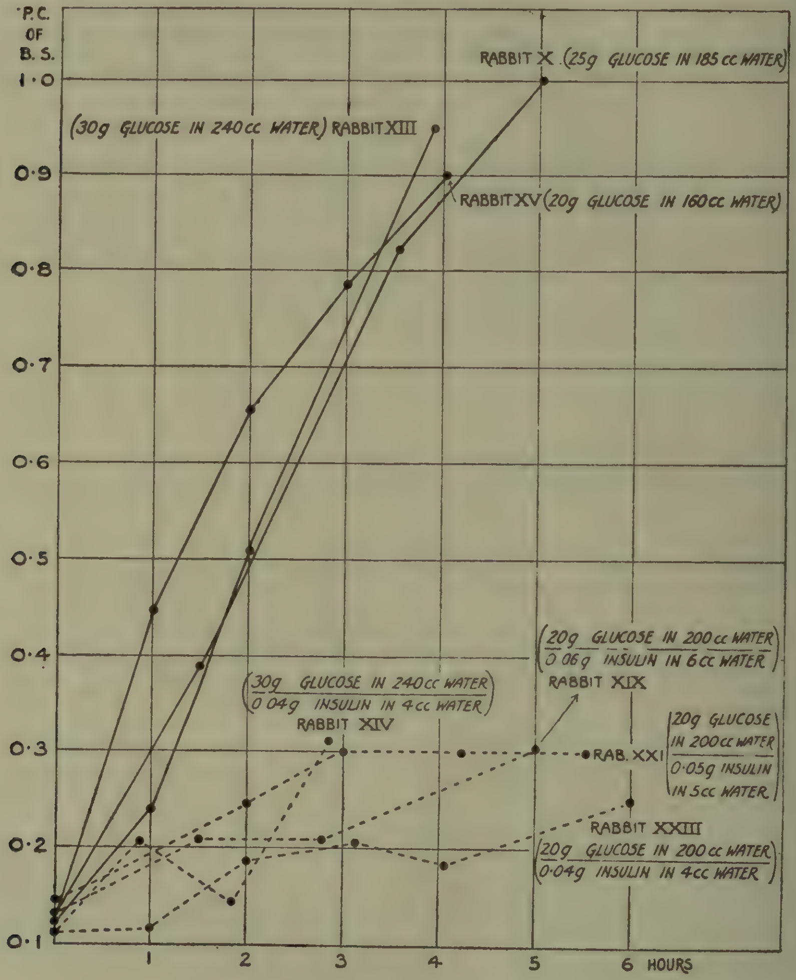

Fig. 1.

All experiments are represented in the form of curves in Fig. 1. It will be seen that insulin always lowers the sugar content of the blood. If glucose is introduced alone, the blood-sugar in a few hours reaches nearly 1 per cent. If insulin is administered simultaneously with glucose, the percentage of blood-sugar does not exceed 0·3 per cent.

Condition of animals.—I always used the insulin (crude or hydrochloride) prepared in the Physiological Institute, University College, London. Testing this preparation (nearly 40 batches) on normal rabbits I often observed convulsions of two kinds: (1) flexion and extension of the whole body with final opisthotonos, and (2) more rarely, rotation of the animal round the longitudinal axis of the body. Neither of these convulsive movements could be observed in the rabbits which received simultaneously glucose and insulin, the amount of insulin being 4 to 5 times greater than in the ordinary test.

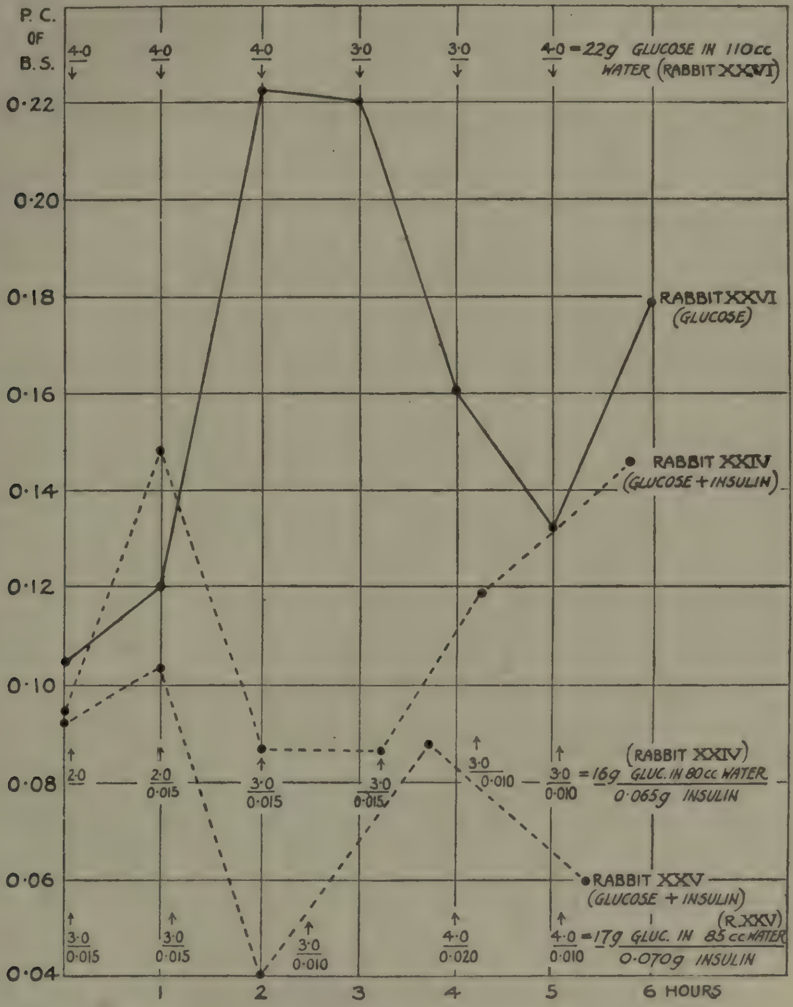

FIG. 2.

In a few experiments the condition of the animals was nearly normal. In other cases, general apathy, weakness and even transitory paralysis of hind legs (in one case) were noticed. This result was not unexpected. The influence of a large quantity of glucose is opposite to that of insulin. Instead of excitement produced by insulin, depression is observed in cases of overloading the blood or the tissues with glucose. The animal is weak, his head drops, he shuts his eyes, his body rocks and he responds feebly to stimulation.

Introduction of glucose and insulin intravenously—In *post-mortem* examination of rabbits which received hypodermic injections of the solution of glucose or glucose and insulin, I observed that a large quantity of fluid remained unabsorbed under the skin. It is possible that insulin creates a condition which may influence the absorption of sugar solution. I repeated these experiments by introducing glucose and insulin intravenously. The solutions were introduced into the vein of one ear; from the vein of the opposite ear samples of blood were taken for blood-sugar estimation. No previous injection of adrenaline was made. The insulin used in these experiments was the same very active preparation as that employed in Experiments XXI and XXIII (see Fig. 1).

The results are represented in Fig 2. The top curve (the unbroken line) shows the blood-sugar content of a rabbit which received an intravenous injection of 2 per cent. glucose solution only (rabbit XXVI). The arrows and figures over it indicate the time and quantity of glucose injected. The two broken line curves are corresponding blood-sugar curves after administration of both glucose and insulin (rabbits XXIV and XXV). The arrows and figures under corresponding curves have the same meaning as the top figures

In both cases insulin lowered the content of blood-sugar. In rabbit XXIV the effect of insulin is not so pronounced as in rabbit XXV. One of the possible causes is the difference in the performance of the experiments: rabbit XXIV received during the first hour an injection of glucose only, rabbit XXV received at once both glucose and insulin. But of course there may be some other unknown reasons for the variable action of insulin, as has been already pointed out by some investigators (McCormick, Macleod, Noble and O'Brien, 1923).

The blood-sugar content after administration of the glucose solution only is not very high (maximum content 0·225 per cent.) This may possibly be due to the dilution of the blood by the solution.

I have only made two observations on the sugar content of the urine. In rabbit XXIV (glucose injected at the beginning of the first hour, insulin at the beginning of the second) and in rabbit XXVI (glucose only) I obtained urine from the bladder after the death of the animals. The estimation of sugar (Benedict's method) gave the following results :

Rabbit XXIV, 29 c.c. of urine with 2·2 per cent. of glucose, or 0·63 gm. glucose secreted.
Rabbit XXVI, 61 c.c. of urine with 2·0 per cent. of glucose, or 1·83 gm. glucose secreted.

It is not possible from these observations to judge the changes of the threshold for glucose in the kidney under the influence of insulin, but they indicate a way in which it is possible to examine these changes.

Influence of Insulin on the Storage of Glycogen.

The question of the storage of glycogen under the influence of insulin in cases of artificial hyperglycæmia is more complicated. The reason is that the glycogen content of the liver and muscles of rabbits, even under uniform conditions to which the animal is submitted, fluctuates. If large doses of adrenaline (1 c.c. or more of 1 : 1000 solution per kilo) are administered the day before, the liver and muscles are practically free from glycogen, but the action of the adrenaline has probably not quite passed off by the next day, when the experiment is performed. This is obvious from the comparatively high blood-sugar content which was noticed in some cases. Moreover, these doses influence the weight of the animal and its capacity to store glycogen, as we shall see later, much more than moderate doses.

On the other hand, smaller doses of adrenaline do not render the liver and muscles quite free from glycogen. But even when the animals were not treated with adrenalin and were fasting before the experiment, the glycogen content of the liver and muscles varied very considerably. All these data are represented in Table II.

We must acknowledge that in this respect the rabbit is not a very suitable experimental subject. Its stomach always contains an unknown quantity of food: the tissue cells are always ready to receive an indefinite quantity of the material for building glycogen. Thus, only very pronounced alterations in glycogen content under experimental conditions could be taken into consideration.

TABLE II. CONTROL EXPERIMENTS.

RABBIT	DATE	WEIGHT IN gm		PER CENT LOSS OF WEIGHT	QUANTITY OF ADRENALINE INJECTED IN CC	PER CENT OF GLYCOGEN			TOTAL WEIGHT OF LIVER	QUANTITY OF GLYCOGEN IN LIVER IN gm.
		DAY BEFORE EXPERIMENT	DAY OF EXPERIMENT			LIVER	MUSCLES	HEART		
I	20th FEB	863	803	6 9	2·0	NO	NO	–	–	–
V	9th MARCH	1963	1792	8·7	4 0	NO	NO	–	–	–
IX	11th APRIL	2264	2116	6·5	3 0	NO	NO	–	99	0
XVII	14th MAY	2828	2711	4·1	2·0	2·96	0·15	0·33	111	3·28
XX	15th JUNE	—	2468	—	NO	0·28	0·13	0·22	67	0·18
XXII	22nd JUNE	1575	1520	3·4	NO	3·84	0·25	–	58·5	2·24

In Table III are represented the experiments with glucose alone or glucose and insulin together. These data do not give us evidence of a more pronounced accumulation of glycogen in the liver and skeletal muscles of rabbits which received glucose along with insulin than in those injected with glucose only, and, even if we assume that a certain storage of glycogen, as compared with control experiments (Table II), occurs, we are able to explain the disappearance from the blood of only a very limited part of the sugar.

The glycogen content of the heart (Tables II and III) undergoes variation which cannot be attributed to the action of insulin (*cf.* Dudley and Marrian, 1923).

Thus these experiments give evidence that insulin does not stimulate appreciably the formation of glycogen in normal animals.

I am much indebted to Prof. E. H. Starling for constant interest and advice during this work.

I wish to express my sincere thanks to Mr. R. K. Cannan for valuable help during the course of these experiments.

TABLE III INJECTION OF GLUCOSE AND INSULIN

RABBIT	DATE	WEIGHT		PER CENT. LOSS OF WEIGHT	QUANTITY OF ADRENALINE INJECTED IN C.C.	QUANTITY OF GLUCOSE INJECTED IN gm	QUANTITY OF INSULIN INJECTED IN gm	PER CENT OF GLYCOGEN			TOTAL WEIGHT OF LIVER IN gm	QUANTITY OF GLYCOGEN IN LIVER IN gm
		DAY BEFORE EXPERIMENT	DAY OF EXPERIMENT					LIVER	MUSCLES	HEART		
IX	22ND MARCH	3038	2649	12·8	3·0	35·0	0·03	0·46	NO	–	99	0·45
XIV	25TH APRIL	2529	2247	11·1	2·5	30·0	0·04	3·07	0·03	0·52	89·5	2·74
XIX	30TH APRIL	2151	1903	11·5	2·5	20·0	0·06	2·36	0·11	0·34	58	1·36
XXI	15TH JUNE	–	3486	–	NO	20·0	0·05	3·33	0·09	0·35	75	2·49
XXIII	22ND JUNE	1971	1878	4·7	NO	20·0	0·04	5·56	0·15	0·28	67	3·72
XXIV	25TH JUNE	–	3269	–	NO	16·0*	0·065*	1·48	0·29	–	102	1·50
XXV	4TH JULY	2859	2728	4·5	NO	17·0*	0·07*	3·05	0·25	–	89	2·71

INJECTION OF GLUCOSE

X	28TH MARCH	2051	1946	5·1	2·0	25·0	–	3·63	0·06	–	72	2·61
XIII	19TH APRIL	2365	2163	8·5	3·0	30·0	–	0·92	TRACES	0·55	64	0·58
XV	8TH MAY	2969	2712	8·6	3·0	20·0	–	0·17	TRACES	0·50	82	0·14
XVIII	29TH MAY	1970	1733	12·0	2·0	20·0	–	1·11	0·06	–	75	0·83
XVI	13TH JULY	2465	2340	5·1	NO	22·0*	–	5·00	0·35	–	98	4·9

* INJECTED INTRAVENOUSLY

The expenses of this research were borne by the Medical Research Council.

SUMMARY.

1. The glycogen content of the liver and skeletal muscles in very well-nourished rabbits, after administration of insulin, is markedly lowered as compared with animals fed in the same way but without the injection of insulin.

2. Insulin always lowers the sugar content of the blood even after subcutaneous or intravenous injection of very large doses of glucose (25–30 gm. in a rabbit of 2–3 kilos).

3. There is no pronounced accumulation of glycogen in the liver and skeletal muscles in rabbits which have received glucose and insulin simultaneously.

4. Insulin has apparently no influence on the glycogen content of the heart.

REFERENCES.

Agadschanianz, R.—(1907) *Biochem. Ztschr.*, **2**, 148.

Banting, F. G., Best, C. H., Collip, J. B., Macleod, J. J. R., and Noble, E. C.—(1922) *Trans. R. Soc. Canada*, Sect. 5, p. 39.

Drummond, W. B., and Paton, D. Noël.—(1904) *J. Physiol.*, **31**, 92.

Dudley, H. W., and Marrian, G. F.—(1923) *Biochem. J.*, **17**, 435.

Maclean, H.—(1919) *Biochem. J.*, **13**, 135.

McCormick, N. A., Macleod, J. J. R., Noble, E. C., and O'Brien, K.—(1923) *J. Physiol.*, **57**, 234.

COMPLEMENT-FIXATION IN STREPTOCOCCAL INFECTIONS.

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THE work to be recorded was undertaken to see how far the complement-fixation test is successful for identifying streptococcal infections. Fixation of complement during union of antigen and specific antibody is the basis of a delicate test for the presence of specific antibodies in the blood, and the presence of these is regarded as important evidence of specific infection.

It was considered that if successful in the case of streptococci, the test might prove to be of great value in the elucidation of the ætiology of rheumatic fever. At present the precise part played by streptococci in rheumatic fever is a matter of controversy. It seemed possible that by employing this serological procedure, one might, for instance, be able to obtain information as to the pathogenic significance of individual strains of streptococci isolated from the patient.

There was also the possibility that the reaction might throw welcome light on certain other conditions of obscure or uncertain ætiology, where a streptococcus has been suspected of causal agency; for instance, that it might help to differentiate between the various types of chronic arthritis.

With these ends in view preliminary observations were made, first to endeavour to see if complement-fixing antibodies are constantly present in streptococcal infections; secondly, to investigate the degree of specificity of these antibodies.

Attention was first given to the results by the complement-fixation test in rabbits prepared with streptococci of known character. During these experiments a routine technique of quantitative character was worked out, and the degree of specificity of the test for differentiating between the various races of streptococci determined. Naturally occurring infection in human beings with streptococci, the characters of which had been definitely ascertained, were studied next. Guided by the results of these preliminary experiments, an attempt was made to find the best method for applying the test to the study of rheumatic fever and allied diseases.

REVIEW OF THE LITERATURE.

There is evidence from previous work that complement-fixing antibodies are present in many streptococcal infections. They have been demonstrated in the serum in cases of scarlet fever (Foix and Mallein, 1907; Schleissner, 1909; Castex, 1909), erysipelas, streptococcal abscess, puerperal fever, streptococcal pleurisy, streptococcal arthritis (Castex, 1909), and "endocarditis lenta" (Major, 1912; Kinsella, 1917). Aschner (1917) found that the serum of a patient suffering from subacute streptococcal endocarditis gave marked complement-fixation with an antigen prepared from the homologous coccus; with a polyvalent antigen prepared from organisms isolated from other cases of malignant endocarditis a positive but less marked fixation occurred.

Attempts to classify the streptococcus group by the complement-fixation test have not been successful. Kinsella and Swift (1917) and Katherine Howell (1918) could not harmonise a grouping by this method with any other classification.

Marked specificity among streptococci has been observed by Floyd and Wolbach (1913–1914) using the complement-fixation method.

The test has been applied to the study of chronic arthritis by Richards (1920). He obtained positive reactions in 68 out of 104 cases, using as antigens mixtures of the strains of *Streptococcus viridans* isolated from the infective foci.

TECHNIQUE.

The reaction was made quantitative by testing the serum in falling dilutions. Details of the technique adopted are summarised as follows:

1. *Hæmolytic system.*—This was adjusted by complement titration, determining the minimum amount of guinea-pig's serum which produces complete hæmolysis of 0·1 c.c. of a 5 per cent. saline suspension of sheep's red corpuscles in presence of the unit of hæmolytic amboceptor and saline to 1 c.c. when incubated in the water-bath at 37° C. for ½ hour.

The unit of hæmolytic amboceptor is twice the minimal dose which completely hæmolyses the unit of sheep's cells in presence of 0·1 c.c. of guinea-pig's serum diluted 1 in 10 and saline to 1 c.c. when incubated in the water-bath at 37° C. for ½ hour.

The unit of sensitised corpuscles to be used in the test as an indicator for complement is thus 0·1 c.c. 5 per cent. suspension of corpuscles *plus* 0·1 c.c. diluted hæmolytic serum containing 2 M.H.D. amboceptor.

The unit of complement used in the test is 0·1 c.c. of diluted guinea-pig's serum containing 1½ or 2 M.H.D. of complement.

2. *Antigen.*—Various preparations of the organism were investigated to determine whether the delicacy of the reaction can be influenced in this way. Very little difference was found between suspensions of the organism grown in different media. A saline suspension of the coccus was slightly more delicate as an antigen if clumps of cocci were removed by light centrifuging. Autolysates, aqueous extract, NaOH extract, the acetone extracted coccus, and this preparation trypsinised for various times, were investigated. None of these gave a more delicate reaction than a simple saline suspension of the coccus. This preparation was used in the experiments to be recorded; the suspension was heated to 60° C. for one hour, and 0·5 per cent. phenol added. Before each test the anti-complementary power of the antigen is determined. Units of complement are incubated at 37° C. for one hour with falling quantities of antigen—viz. 0·1 c.c. doses containing respectively 400, 200, 100 and 50 million cocci. The unit of sensitised corpuscles is then added to each tube, and after further incubation for ½ hour the anti-complementary dose is known. When this dose is one which causes a trace only of inhibition of hæmolysis, half the quantity is used in the test; in other cases one quarter the anti-complementary dose is used.

3. *The test.*—Falling amounts of inactivated serum—1/100 c.c., 1/200 c.c., 1/400 c.c., etc.—are incubated at 37° C. for 1 hour with 1½ or 2 M.H.D. of complement, and ½ or ¼ the anti-complementary dose of antigen. More precisely each tube contains 0·1 c.c. diluted guinea-pig's serum containing 1½ or 2 M.H.D. of complement, 0·1 c.c. requisite dilution of

antigen, and 0·1 c.c. serum diluted 1 in 10, 1 in 20, 1 in 40, etc. In addition the following control tubes are put up:

1. Serum control:
   0·1 c.c. serum diluted 1 in 10, 1 in 20, etc., each with the unit of complement.
2. Antigen control:
   The dose of antigen used in the test, with the unit of complement.

After this incubation the unit of sensitised corpuscles is added, and the total volume in each tube made up to 1 c.c. with saline. After a further incubation at 37° C. for ½ hour the results are read off. A range of serum dilutions sufficient to bring out the end-point is tested. The final dilution of serum in each tube is 1 in 100, 1 in 200, 1 in 400, etc., and the actual amount of serum present in each tube is 1/100 c.c., 1/200 c.c., etc.

OBSERVATIONS WITH SERA FROM ARTIFICIALLY IMMUNISED RABBITS.

Before investigating naturally occurring infections in the human being, it was desirable to determine the reaction of an animal when treated with a known type of streptococcus. The object of these experiments was to see if complement-fixing antibodies can be detected in the serum of such an artificially immunised animal, and to what extent they can be produced. Further, one wished to determine the behaviour as antigens of the three main groups of streptococcus—*pyogenes*, *salivarius* and *fæcalis*—particularly as regards the degree of specificity of the antibodies produced by typical members of each group.

Three rabbits were immunised by Dr. M. H. Gordon, one with *Streptococcus pyogenes*, Type I (Gordon, 1921), the second with a typical example of the *Streptococcus salivarius* group, the third with a typical example of the *Streptococcus fæcalis* group. After 12 days, during which the animals were given five injections, samples of serum were taken and tested. Injections were intravenous. The *pyogenes* animal received an initial dose of 250 millions, increased to 2000 millions in 12 days; initial doses for the *salivarius* and *fæcalis* animals were 500 millions, the dosage again being increased to 2000 millions in 12 days. Further samples were tested at intervals during a period of 2½ months, immunisation being continued over this time, the dose being increased in each case to 16,000 millions.

Each sample was tested with the three antigens under investigation; the result with normal rabbit serum and these antigens was negative in each case. The first samples of serum from the *salivarius* and *fæcalis* rabbits gave negative reactions with all three antigens, and by testing at intervals the increase in complement-fixing antibodies could be observed.

The information obtained from these experiments may be summarised as follows:

(1) Rabbits treated with typical examples of the *pyogenes*, *salivarius*, and *fæcalis* groups respond by production of complement-fixing antibodies in sufficient quantity to be readily demonstrated by the technique adopted.

(2) Complement-fixing antibodies appear to be more rapidly formed to *S. salivarius* and *S. fæcalis* than to *S. pyogenes*. The highest titre with the *salivarius* and *fæcalis* rabbits—1 in 800 and 1 in 3200 respectively—was obtained on the eighteenth day; the highest titre with the *pyogenes* rabbit—1 in 1600—was obtained after two months.

(3) *Pyogenes* serum shows little specificity for the homologous coccus. The serum was tested with various heterologous cocci of the *salivarius* and *fæcalis* groups, and in all cases fixation was obtained, in the majority to the same titre as that of the homologous reaction.

(4) A definite specificity for the homologous coccus is observable in both

salivarius and *fæcalis* sera; in both cross-fixation occurs with the examples of the other groups tested, but this is to a lower titre than the homologous fixation.

OBSERVATIONS IN HUMAN INFECTIONS.

(A) *Infection with Non-hæmolytic Streptococci.*

Malignant endocarditis affords a good example of infection with the non-hæmolytic group of streptococci. Ten examples of this infection were investigated; in each case the infecting coccus was isolated by blood-culture. Seven were *Streptococcus salivarius* infections, three *Streptococcus fæcalis*; all were of some months' duration.

The patient's serum was tested with the homologous coccus and with heterologous cocci (1) from the other cases investigated as they became available, (2) isolated previously by Dr. M. H. Gordon from similar cases. All these cocci were shown by Dr. Gordon to be serologically distinct when tested by the method of agglutination and absorption of agglutinin. Negative controls in each case were satisfactory, the reaction of these antigens with normal human serum being negative at 1 in 100.

In all cases the patient's serum gave a positive reaction with the homologous coccus. The end-point varied from 1 in 800 to 1 in 6400. The serum showed a high degree of specificity for the homologous coccus. No cross fixation at all was demonstrable with some of the heterologous cocci tried; with others cross-fixation occurred, both with cocci belonging to the same group as the homologous coccus and with examples of the other groups, though this cross-fixation was to a much lower titre than the homologous fixation.

A typical example of the results obtained in these cases is shown in the following table:

Case: Andrews. S. salivarius *isolated by Blood Culture.*

Antigens.		Andrews serum. 1 in—							
		100	200	400	800	1600	3200	4800	6400
Salivarius	Andrews	++	++	++	++	++	++	+	–
	Ross	++	++	+	+–	–			
	Day	+	+–	–	–	–			
	Hodson	+	+–	–	–	–			
	Magona	+	–	–	–	–			
Fæcalis	Bradshaw	+	+–	–	–	–			
	Harvey	+–	–	–	–	–			
Pyogenes Type 1	155	++	++	+–	–	–			

++ = Complete inhibition of hæmolysis.
+ = Between complete inhibition and 50% inhibition.
+– = Between 50% inhibition and complete hæmolysis.
– = Complete hæmolysis.

It was observed in these cases that fixation with a heterologous coccus of the same group as the homologous coccus (*i. e. salivarius* or *fæcalis*) may be no greater or even less than the cross-fixation with a member of another group.

The maximum amount of complement fixed by 1/100 c.c. of serum from one of these cases—a *Streptococcus fæcalis* infection—was determined. The end-point with 1½ M.H.D. was 1 in 1600; by testing with increasing doses of complement it was proved that 1/100 c.c. of serum fixed at least 3½ M.H.D.

(Note: the sera of these cases of malignant endocarditis and the rabbits' sera discussed in the last section were tested by methods of agglutination and absorption of agglutinin by Dr. M. H. Gordon.)

(B) *Infection with Hæmolytic Streptococci.*

The results obtained in eight of these infections are recorded. All cases were tested with a stock *S. pyogenes*, Type I, and five were also tested with an antigen prepared from the actual infecting coccus. In each case a known *pyogenes* serum (rabbit) was put up as a control and was satisfactory. These observations indicate that the serum from a case of *S. pyogenes* infection is poor in complement-fixing antibodies—a finding not unexpected, as these infections are acute and of comparatively short duration.

CASES 1, 2 AND 3.—Erysipelas. 12 days', 3 weeks', and 8 weeks' duration respectively. (Axillary abscess in case 3.) Reaction in each case negative at a serum dilution of 1 in 50.

CASE 4.—Cellulitis. 4 weeks' duration. Reaction negative at 1 in 50.

CASE 5.—Acute mastoiditis. 2 weeks' duration. Reaction with *S. pyogenes*, Type I, positive to 1 in 200. Antibodies disappeared with recovery.

CASE 6.—Mastoid abscess. 17 days' duration. Reaction negative at 1 in 50.

CASE 7.—Septicæmia. 10 weeks' duration. Reaction with infecting coccus positive to 1 in 400, with a representative of the *salivarius* group negative at 1 in 50, and with a typical *S. fæcalis*, positive to 1 in 100. The serum appears more specific than that of the artificially immunised rabbit.

CASE 8.—Suppurative arthritis. 10 days' duration. Reaction positive to 1 in 50.

A CONSIDERATION OF THE POSSIBILITY OF IDENTIFYING STREPTOCOCCAL INFECTIONS BY THE COMPLEMENT-FIXATION TEST.

Dr. Gordon's (1922) observations with agglutinin have shown that while the hæmolytic streptococci comprise three serological types only, the non-hæmolytic group is divisible into extremely numerous types, which exhibit serologically (agglutinin) a high degree of specificity.

Results so far recorded suggest that in *salivarius* or *fæcalis* infections of some months' duration, complement-fixing antibodies to the infecting coccus would be demonstrable in the patient's serum. The fact that a positive reaction is not usually obtained in the acute conditions caused by *S. pyogenes* does not lessen the possible value of the test; diagnosis in such cases is not usually a matter of difficulty.

Two possible methods suggest themselves for applying this test to the investigation of cases suspected on clinical grounds to be streptococcal infections.

First the use of polyvalent antigens may give evidence of streptococcal infection, though on account of the large number of highly specific types which make up the non-hæmolytic group, it is obvious that little value can be attached to a negative reaction. This procedure has been tried in a number of cases where, clinically, a streptococcus was suspected of causal agency, including cases diagnosed as infective endocarditis, with negative blood cultures, iritis, rheumatic fever, chronic arthritis. The antigens used, viz. *S. pyogenes*, Type I, polyvalent *S. salivarius*, and polyvalent *S. fæcalis*, have been tested with normal human sera, and shown to give negative reactions at a serum dilution of 1 in 50 in each case (seven normal sera were tested). The large number of negative results obtained served as further negative controls, and it would therefore appear probable that a well-marked positive reaction can be regarded as indicating streptococcal infection. But the method is unsatisfactory, because at present representative antigens cannot be made, and consequently infection cannot be excluded by a negative result.

Out of five cases diagnosed clinically as malignant endocarditis, but which gave negative blood cultures, only two gave a positive reaction, and in only one of these was the reaction well marked. Of twenty cases of chronic arthritis a well-marked positive reaction was obtained in seven, a doubtful reaction (weak positive) in seven, and a definitely negative reaction in six.

The second method is to test the patient's serum with individual strains of streptococci isolated from possible infective foci—a tooth-socket, a tonsil, or the intestinal tract; or in cases of rheumatic fever, with streptococci isolated from various organs and body-fluids either during life or *post mortem*.

The question of the *rôle* of streptococci in the ætiology of rheumatic fever remains unanswered. The view has been repeatedly expressed that rheumatic fever is a specific bacterial infection, and that the organism is a streptococcus of the non-hæmolytic group. It is hoped that further evidence as to the pathogenic significance of streptococci isolated from various organs and body-fluids in cases of rheumatic fever and chorea may be obtained by applying the complement-fixation test for the detection of specific antibodies in the patient's serum. The study of rheumatic fever along these lines is in the early stages; the number of cases and controls investigated is not sufficient for any definite statement to be made. A brief record, however, of the work carried out so far may perhaps be added. As the tonsil has been suspected of being the site of invasion, it seemed best to investigate, first, the problem as to whether or not a streptococcus giving a positive reaction with the patient's serum can be isolated from the tonsil. Cases were therefore studied in this way as opportunity offered; several strains of streptococci were isolated from tonsil cultures, and each strain tested with the patient's serum. Cultures were aërobic, and on trypagar enriched with blood or ascites fluid. Of seven cases so investigated, positive results were obtained in three:

(1) Rheumatic heart disease, subcutaneous nodules; duration 3 months.

Reaction of serum positive with 1 out of 7 streptococcus colonies isolated, a *S. pyogenes*, giving an end-point of 1 in 1600. (Agglutination test, by Dr. M. H. Gordon, positive to 1 in 200 with this coccus.)

(2) Rheumatic fever. Duration 6 weeks. Reaction positive with 1 out of 19 streptococcus colonies isolated; this organism gave the carbohydrate reactions of *S. salivarius*; end-point 1 in 400.

(3) Rheumatic fever. Duration 5 months. Reaction positive with 1 out of 18 colonies, this organism giving the carbohydrate reactions of *S. pyogenes*; end-point 1 in 200.

In the other four cases, including two of acute rheumatism, a streptococcus giving a positive reaction with the patient's serum could not be isolated from the tonsil.

The number of streptococcus strains isolated which gave a negative reaction is striking, and suggests the possibility that the cocci giving positive reactions are heterologous organisms having a saprophytic existence on the tonsil.

It is suggested that the complement-fixation test should prove of value in the experimental study of rheumatic fever and allied diseases.

SUMMARY.

(1) If the complement-fixation test is successful for identifying streptococcal infections, it might prove of value in the experimental study of rheumatic fever and allied diseases. The observations recorded were carried out to see how far the reaction is of value for this purpose.

(2) Rabbits respond to treatment with typical examples of the three main groups of streptococci by production of complement-fixing antibodies in sufficient quantities to be easily demonstrable. A definite specificity is observable in both *salivarius* and *fæcalis* sera, but a *pyogenes* serum shows little specificity for the homologous coccus.

(3) Ten cases of infective endocarditis were investigated. In all the patient's serum gave a positive reaction, to a high titre, with the homologous coccus (obtained by blood culture); a high degree of specificity is observable, though cross-fixation may occur with a streptococcus of any group. The groups *pyogenes*, *salivarius* and *fæcalis* are not distinguishable by complement-fixing antibodies.

(4) A positive reaction is not usually obtained in the acute conditions caused by *Streptococcus pyogenes*.

(5) Two methods of applying the test to the experimental study of rheumatic fever and allied diseases are suggested, viz.:

a. By using polyvalent antigens.

b. By testing the patient's serum with individual strains of streptococci, isolated from possible infective foci, blood, pericardial fluid, joint fluid, etc.

The second procedure appears, at present, to offer the best means of applying the test to obtain further evidence as to the *rôle* of streptococci in these diseases. It is suggested that, in cases of some months' duration, the complement-fixation test affords a reliable criterion as to the pathogenic significance of a non-hæmolytic streptococcus isolated from the patient.

The work recorded is part of a research on the streptococcus group which is being carried out for the Medical Research Council in the Pathological Department of St. Bartholomew's Hospital.

REFERENCES.

ASCHNER, P. W.—(1917) *J. Infect. Dis.*, **21**, 409.
CASTEX, M. R.—(1909) *Presse méd.*, **17**, 324.
FLOYD, C., AND WOLBACH, S. B.—(1913–14) *J. Med. Res.*, **29**, 493.
FOIX, C., AND MALLEIN, E.—(1907) *Presse méd.*, **15**, 777.
GORDON, M. H.—(1921) *Brit. Med. J.*, **1**, 632.—(1922) *J. State Med.*, **30**, 432.
HOWELL, KATHERINE.—(1918) *J. Infect. Dis.*, **22**, 230.
KINSELLA, R. A.—(1917) *Arch. Int. Med.*, **19**, 367.
KINSELLA, R. A., AND SWIFT, H. F.—(1917) *J. Exp. Med.*, **25**, 877.
MAJOR, R. H.—(1912) *Bull. Johns Hopkins Hosp.*, **23**, 326.
RICHARDS, J. H.—(1920) *J. Bact.*, **5**, 511.
SCHLEISSNER, F.—(1909) *Wien. klin. Wochenschr.*, **22**, 553.

SOME CULTURAL CHARACTERISTICS EXHIBITED BY SEROLOGICAL TYPES OF MENINGOCOCCI.

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As early as 1909 Arkwright found some evidence that different strains of meningococcus fall into various groups as regards their agglutination reactions. He also found differences in their power of fermentation of glucose and maltose. Martin (1910) discovered that some meningococcus strains ferment dextrose less rapidly than maltose, while Griffith (1916), on the basis of simple agglutination tests, divided 34 cerebro-spinal strains from an epidemic into two main groups which, with few exceptions, exhibited corresponding fermentative properties, namely, (1) strains which fermented maltose more than glucose, and (2) strains which fermented glucose more than maltose. In 1915, on the basis of agglutination tests, Gordon and Murray (1915) proposed the division of these organisms into four types. Opinion is divided as to the justification of this grouping, and many authors consider that there is so much affinity between their types I and III on the one hand, and II and IV on the other, that it is more rational to recognise two main types only. Continental workers have always insisted on two broad groups—for example, the meningococcus and parameningococcus of the French writers. Hitherto no serious attempts have been made to correlate these types with cultural characteristics, or to associate the appearances of cultivations of different strains of this organism with any other definite grouping. Thus the following passage occurs in a paper by Nicolle, Debains, and Jouan (1918): "Considérés au point de vue de leurs 'caractères généraux' (morphologie, aspect des cultures, propriétés biologiques), les méningocoques offrent des traits communs, que chacun connaît aujourd'hui et des différences individuelles. Ces différences, légères et sans relation entre elles, ne permettent pas de créer des groupes distincts. Nous allons montrer que les méningococciques, envisagés au point de vue de leurs 'caractères antigènes' manifestent, par contre, des dissemblances marquées et concordantes, imposant l'idée de type tranchés."

Meningococcus agglutinating serum for four types is laborious to prepare, and as there is not a great demand for it, especially during the summer months, it is often difficult to obtain. Moreover, the accurate typing by agglutinating sera is an intricate process requiring considerable care. Many strains when first isolated are practically inagglutinable, and some, even after cultivation on artificial media for some time, are only agglutinated by a serum at several times its titre-strength, which has led not a few observers to class a strain as not belonging to any of Gordon's four types. The titres of the type sera must be accurately known, otherwise we may give undue weight to group agglutination, which sometimes occurs. When sera are employed in greater concentrations than their titre-strength we must be alive to the possible presence of "zone" effects which may completely invert the typing. To an experienced hand this presents no real difficulty, but it may frequently fall to the lot of one not specially versed in the complexities of agglutination to have to assign its type to a newly isolated strain. It would therefore be convenient to have a set of cultural tests, if possible, by which the types could be distinguished whenever a strain is isolated from the spinal fluid. By a modification of technique I have succeeded in obtaining quite marked cultural differences, especially in the case of the two main types, which agree remarkably well with the serological grouping.

METHOD OF EXPERIMENT.

While casting round for a medium which should at the same time be cheap and favourable for the growth of the meningococcus, Gordon decided upon one made by dissolving agar in trypsinised broth and adding a saline extract of peaflour. This is known as "trypagar" (trypsin-broth-pea-agar). This is the medium chiefly used in the present work. Its preparation is as follows:

Mince 1 lb. of meat and boil in 1 litre of water; filter, and make faintly alkaline to litmus; heat at 85° for five minutes and then cool to 37°; add 1 per cent. trypsin and keep at 37° for three hours; make slightly acid and boil for twenty minutes. After standing overnight syphon off the clear fluid; this is called "trypbroth." Take 1 litre of trypbroth and add 2 per cent. agar; dissolve and make slightly alkaline; cool, add egg for clearing, autoclave and filter; add 5 per cent. peaflour extract; steam on two successive days for twenty minutes.

The peaflour extract is made by grinding a 10 per cent. emulsion of peaflour in 10 per cent. NaCl in a mortar. It is then steamed for twenty minutes and filtered.

It was observed that on this medium some of the stock strains tended to die out sooner than others—a circumstance which suggested a thorough investigation to discover whether this feature could be made the basis of a method of typing meningococcus cultures.

Petri plates of about 9 cm. diameter were filled with trypagar to a depth of 7–9 mm. This is considerably more medium than is usually employed for plate cultures, but it is necessary for uniform results. In making these plates contamination occurs very readily and certain special precautions are necessary. Owing to the relatively large amount of medium in each plate, a good deal of condensation water forms on the lid when the plates get cold. This can be removed by carefully lifting the lid slightly and tilting, which causes the drops of water to run off at the side. If this is not attended to the sides of the plate remain very moist, and the water soon absorbs enough nutritive material from the medium to enable air organisms to grow round the edges. After pouring, the plates were placed in the incubator at 37° for a day or two to free the surface of the medium from loose water, and incidentally, also, to ensure their sterility. They were then turned upside down, and a circle whose diameter was 5·2 cm. was inscribed on the glass concentric with the plate. Six equidistant points were marked on this circle as the sites of the inoculations. In this way any slight irregularities in the plate that happened to be present were largely counteracted, as they mostly consist of concentric ripples, and a medium of uniform thickness was obtained. The two strains of meningococcus to be tested were inoculated from overnight cultures alternately by just touching the six points with the charged platinum needle. The appearance of the plate is shown in Figs. 2 and 3. They were then incubated upside down at 37°. After they had ceased growing—usually in 6 or 7 days—small samples were taken from the edge of the colony from day to day and inoculated on trypagar slopes to see whether the colony was still viable. A fresh bit of the edge was used each time, because it was necessary to avoid any portions which may have been pushed further out into the medium on a previous occasion and thus have been enabled to grow afresh, with, consequently, an extended lease of life. It was found on trial that the centre of the colony did not live longer than the edge; in fact it usually died first. It was essential in the first instance to see how different strains of the same serological type behaved.

The strains used in this work, numbering about 30, came from several different countries, and although not large in number they may be taken as fairly representative of the disease in various parts of the world. They were all spinal in origin. Some of them were Gordon's original strains as received from the Royal Army Medical College; others were obtained from other parts of Great Britain, Denmark, Poland, South Africa and the United States. They had been cultivated on artificial media for variable periods ranging from the early part of the war up to the present time. There is no evidence that any of them have changed their serological type since isolation. Most of them have been tested several times.

VIABILITY TESTS.

In Table I two strains* of each type are compared with one another as regards length of life on trypagar plates. As stated above, alternate colonies are from the same strain. It will be noticed that the variations which occur are about as great between colonies of the same strain as between different strains of the same type. These results are quite representative of many tests that have been made. Occasionally anomalous results have been obtained where one or two colonies have long outlived the rest owing to the medium having separated from the opposite side of the plate and shrunk on account of drying, but where no unusual happening of this nature has taken place the results have been quite uniform. The widest variations are 3 or 4 days. Sometimes the sample taken on a certain day fails to grow, although a subsequent sample shows that the whole of the colony is not dead. This usually presages the end. Several negative tests had to be obtained before finally concluding that the colony was dead. Where a blank space is left the colony was not tested on the day in question.

TABLE I.

COMPARISON OF THE LENGTH OF LIFE OF DIFFERENT STRAINS OF THE SAME TYPE ON TRYPAGAR.

STRAINS DAYS AFTER INOCULATION

LITTLEDALE(I) v. HOWES(I)	8	9	10	111	12	13	14	15
1	+	+	+	+	O	O	O	
2	+	+	O	+	O	O	O	
3	+	+	+	+	+	O	O	O
4	+	+	+	O	O	O		
5	+	+	+	+	O	O	O	
6	+	+	O	O	O			

PUGH (II) v. RAD (II)	8	9	10	11	12	13	14	15	16
1	+	+	+	+	+	O	O	O	
2	+	+	+	+	+	+	O	O	O
3	+	+	+	+	+	O	O	O	
4	+	+	+	+	+	O	O	O	
5	+	+	+	+	+	O	O	O	
6	+	+	+	+	+	+	O	O	O

CHASE (III) v. R.F. (III)	8	9	10	11	12	13	14	15	16	17
1	+	O	+	O	O	O				
2	+	+	O	O	+		+	O	O	O
3	+	O	+	O	O	O				
4	+	+	+	+	+	O	O	O		
5	+	+	+	O	+	O	+	O	O	O
6	+	+	+	O	O	O				

TAPLOW(IV) v. BUTTERY(IV)	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1		+	+		+	+	+	+		O		O	O	O	
2		+	+		+	+		+		O		O	O	O	
3		+	+		+	+	+	+		O		O	O	O	
4		+	O		+	+		+		+		O	O	O	O
5		+	O		+	O	O	+		O		O	O	O	
6		+	+		+	+		+		+		O	O	O	O

Turning now to a comparison of strains of different types, Table II shows that there is a large difference between types I and II, the former usually dying out much earlier. The same distinction is found between types I and III, in favour of a considerable longer life for III. The difference between II and III is not nearly so marked, and it is doubtful whether any conclusion can be drawn as to any advantage in viability possessed by one of these types over the other. The same applies to types II and IV.

The last three experiments in Table II are anomalous and deal with two strains, Sp 38 and 46 B, of rather uncertain type. More will be said about these later.

It was interesting now to enquire whether the same distinction between types I and II obtained on a serum-agar medium. The basis of this medium

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* In this paper each strain is referred to by name, and its type, in conformity with Gordon's grouping, is appended in brackets.

TABLE II

COMPARISON OF THE LENGTH OF LIFE OF DIFFERENT TYPES ON TRYPAGAR.

TYPES DAYS AFTER INOCULATION

PUGH (II) v. S. A. (I)	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1					+	+	+	+	+	+		+		+		+	+		+		○	○	○	○
2	+		+		○	○	○	○																
3					+	+	+	+	+	+		+		+		+	+		○		○	○	○	
4	+		○		○	+	○	○	○	○														
5					+	+	+	+	+	+		+		+		+	+		○		○	○	○	
6	+		○		○	○	○																	

PUGH (II) v. LITTLEDALE (I)	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	+		+	+	+	+	+	+	+	+	+	+	+	○	○	○	
2	+		○	○	○	○											
3	+		+	+	+	+	+	+	+	+	+	+	+	+	○	○	○
4	+		○	○	○	○											
5	+		+	+	+	+	+	+	+	+	+	○	+	○	○	○	
6	+		○	○	○	○											

PUGH (II) v. AM. (I)	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+	+	+	+	+	+	+	○	○	○			
2	+	+	+	○	○	○							
3	+	+	○	+	+	+	+	+	+	+	○	○	○
4	+	+	+	+	○	○	○						
5	+	+	+	+	+	+	○	○	○				
6	+	+	○	○	○	○							

GABBITAS (II) v. HOWES (I)	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	+		+	+	+	+	+	+	+	+	+	○	○	○	○	
2	+		○	○	○	○										
3	+		+	+	+	+	+	+	+	+	+	+	+	○	○	○
4	+		○	○	○	○										
5	+		+	+	+	+	+	+	+	+	+	+	○	○	○	
6	+		○	○	○	○										

PUGH (II) v CHASE (III)	10	11	12	13	14	15	16	17	18	19	20	21	22
1	+		○	○	○	+		○	○	○	○		
2	+		+	+	+	+	+	+	+	○	○	○	○
3	+		+	+	○	+	○	+	○	○	○	○	
4	+		+	+	+	+	+	+	+	○	○	○	
5	+		+	+	○	+	○	○	○	○			
6	+		+	+	+	+	+	+	+	○	○	○	○

HOWES (I) v. CHASE (III)	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	+	+	○		○	○	○	○	○										
2	+	+	○		+	+	+	+	+	+		+		○	○	○	○		
3	+	○	○		○	○	○	○	○										
4	+	○	○		+	+	+	+	+	○		+		+	+	○	○		○
5	+	○	○		○	○	○	○	○										
6	+	+	○		+	+	+	+	+	+		+		○	○	○	○		

TABLE II (CONTINUED)

TYPES	DAYS AFTER INOCULATION												
PUGH (II) v. AM. (III)	18	19	20	21	22	23	24	25	26	27	28	29	30
1	+	+	+	+	+	+	+	O	O	O			
2	+	+	+	+	+	O	+	+	+	O	O	O	O
3	+	+	O	+	+	+	O	O	O	O			
4	+	+	+	+	+	O	+	O	O	+		O	O
5	+	+	+	+	+	O	O	O					
6	+	+	+	+	+	+	O	O	O	O			

PUGH (II) v. TAPLOW (IV)	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1					+	O	+	+	O	O		O	O	
2	+		+		O	O	O	+	O	O		O	O	
3					O	+	O	+	O	O		O	O	
4	+		O		O	+	+	+	O	O		O	O	
5					+	+	+	+	+	O		O	O	O
6	+		O		+	+	O	O	O	O		O	O	

PUGH (II) v. HICKS (IV)	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	+	+	+	+	+	+		+	+	O	O	O	O				
2	+	O	+	O	O	+		O	O	O	O	O					
3	+	O	+	+	+	+		+	+	O	O	O	O				
4	O	+	O	+	+	O		O	O	O	O	O					
5	+	+	O	+	+	+		+	+	O	O	+	O		O	O	O
6	O	O	O	+	O	+		+	+	O	O	O	O				

PUGH (II) v SP. 38 (II)	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+			+	O	O	+	+	+		O	+	+	+	O
2	+	+		O	O	O	O								
3	+			+	+	+	O	+	+		+	+	O	O	O
4	+	O		O	O	O	O								
5	+			+	+	O	+	+	+		+	+	+	+	O
6	+	+		O	O	O	O								

PUGH (II) v. 46 B (II)	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
1	+			+		+		+			+		+			+		+		+		+		+			O		O			O		O
2	+	+	+	O	O	O		O																										
3	+			+		+		+			+		+			+		+		+		+		+			+		O			+		O
4	+	+	+	O	O	O		O																										
5	+			+		+		+			+		+			+		+		+		+		+			+		+			O		O
6	+	+	O	O	O	O		O																										

LITTLEDALE (I) v. SP. 38 (II)	7	8	9	10	11	12	13	14
1	+		+		O	O	O	O
2	+		+		O	O	O	O
3	+		+		O	O	O	O
4	+		+		O	O	O	O
5	+		+		O	O	O	O
6	+		O		O	O	O	O

was trypsinised meat broth with an addition of horse-serum of about 1 in 4. Table III shows that there is practically no difference in the length of life of the two types mentioned on this medium, which proves, among other things, that it can hardly be different powers of resistance to drying *per se* that account for the differences on trypagar.

To sum up, these viability tests on trypagar show a clear distinction between type I and the other three types, in favour of a considerably shorter life for the former. In many cases it survives less than half the time that the others do. Between types II, III and IV there are no such clearly defined differences, and it is hardly possible to separate them by this test. The viability on a serum-agar medium, on the other hand, is about the same for types I and II. The relatively short life of type I on trypagar may be due to the formation of larger amounts of some decomposition product, possibly the alkali produced.

TABLE III.

COMPARISON OF THE LENGTH OF LIFE OF TYPES I & II ON SERUM AGAR.

TYPES PUGH (II)	V. S. A (I)	8	9	10	11	12	13	14	15	16				
1		+	+	+	+		O	O	O	O				
	2	+	+	+	+		O	O	O	O				
3		+	+	+	+		O	O	O	O				
	4	+	+	+	+		O	O	O	O				
5		+	+	+	+		O	O	O	O				
	6	+	+	+	+		O	O	O	O				

PUGH (II)	V LITTLEDALE (I)	6	7	8	9	10	11	12	13	14	15	16	17	18
1		+	+	O	+		+		O	O	O	O		
	2	+	O	+	O		+		O	O	O	O		
3		+	+	+	O		+		O	O	O	O		
	4	+	+	O	+		O		O	+	O	O		O
5		+	+	+	+		+		O	O	O	O		
	6	+	+	+	+		+		O	O	O	O		

APPEARANCES OF COLONIES.

The cultural features now to be described were obtained on plates of trypagar similar to those employed above. By virtue of the thickness of the medium (7–9 mm.), the colonies were able to grow to a large size and develop characteristic surfaces which are not observed when growth is checked at an earlier stage. This seems to be the reason that they have not been described before, for although the medium is not new, the method of employing it in a much thicker layer in plates does not appear to have been tried previously.

The strains of type I again show the greatest individuality. This type grows as a whitish or pale yellowish colony with a more or less scalloped or indented border. From the beginning the surface is usually somewhat rough and may be intersected by furrows. About the time the colony attains its full size papillæ begin to appear in most cases, scattered all over the surface. Some are larger than others, but occasionally the entire surface is covered with *minute* papillæ. The time of their appearance varies a good deal, as it depends upon the rate of growth of the colony. They are presumably an expression of the reaction of the organism against one of its own metabolic products—perhaps the relatively strong alkalinity produced in the medium. About this time, or a little later, a halo begins to form round the colony which gradually extends outwards in the medium, often eventually taking the form of a ring surrounding the colony. The formation of this halo is probably caused by the precipitation of a salt of legumin by the alkali formed. The legumin is derived from the peaflour and although said to be itself soluble, its salts are considered to be insoluble (Osborne, 1909). The above features are well seen in Fig. 1, which is a single colony, grown on a plate of trypagar, of a South African strain of type I (S.A. (I)), and also in Fig. 7, which illustrates a colony of each of the four types.

Type II is well contrasted with this. It grows as a slightly smaller round colony with a much more even edge. The surface is very smooth and glistening. No papillæ are produced. The colony soon begins to show evidence of the development of a yellow pigment which tends to deepen in colour as the colony attains its full size. This characteristic alone is usually sufficient to distinguish it from types I and III. No halo is formed.

Type III resembles type I in many respects. The colour of its colony is whitish, or sometimes pinkish in the thick parts. The characteristic appearance of its surface may be described as "lumpy," which is well portrayed in Fig. 2. Papillæ usually do not develop on its surface and there is no halo. Some strains of type III, however, do show papillæ on the surface of the colony, notably a Danish strain, 15 (III), in my collection (Fig. 3). Such a strain seems to be an intermediate one between typical examples of types I and III.

Type IV greatly resembles type II—in fact there are practically no distinguishing marks in the colonies of these two types. Wadsworth, Gilbert and Hutton (1920) suggest that type IV, may not be a separate group, but possibly a poorly agglutinating II or III. They made some agglutinating serum from a strain of type IV obtained from Gordon which agglutinated strains of type II much more than its homologous strain. At all events there seems to be considerably less difference between types II and IV than between types I and III.

It may be useful to tabulate the above characteristics of the different types.

TABLE IV.—*Appearance of Colonies of the Different Types on Thick Trypagar Plates.*

Type.	Shape.	Size.	Colour.	Surface.	Papillæ.	Halo.
I	Irregular	Larger	Whitish or pale yellowish	Rough	+	+
II	Round	Smaller	Yellow	Smooth, glistening	0	0
III	Irregular	Larger	Whitish or pinkish	Lumpy	+ or 0	0
IV	Round	Smaller	Yellow	Smooth, glistening	0	0

As stated above, the development of these characteristics depends upon the colony having sufficient space for its complete evolution. Fig. 4 illustrates this point very distinctly. Colonies of W.30.B. (I) and Pugh (II) have been planted on the usual thick trypagar plate but they are more crowded than before, with the result that growth has been checked from lack of nourishment before the distinguishing features (rough surface, papillæ and halo in type I) could develop. The yellow pigment in the case of type II, however, could be detected, as this is an early character to appear.

On thick glucose trypsin-broth agar the pigment formation is more marked. Colonies of type I are usually pure white; type II assumes a deep yellow or even reddish-brown tint; type III had a pinkish or purplish tint; and type IV much resembles type II, although it is possibly more inclined to be yellow. Fig. 5 shows the appearance of the four types on this medium. The almost snow-white colour of S.A. (I) can be visualised from the photograph. Contrary to what occurs on trypagar, it will be observed that Pugh (II) develops papillæ on this medium probably in response to some decomposition product of the fermented glucose.

On ordinary trypsin-broth agar papillæ do not occur on the colonies of any of the types, nor does type I produce a halo, which is in accordance with the hypothesis propounded above. There is, however, some difference between types I and III and types II and IV as a glance at Fig. 6 will show. The colonies have much the same appearance on serum agar.

It is not insisted that the characteristic appearances defined for the different types of meningococcus form hard and fast boundaries between them. It would indeed be remarkable if this were so in organisms which must be so closely related as the types of meningococcus. Thus a few papillæ may very occasionally form on type II colonies growing on trypagar. The halo described appears to be a diffusion outwards of a turbidity regularly occurring in the medium directly beneath the colonies of all the types. In plate cultures which had been stored for a week or two in the cold room prior to being photographed, it was noticed that even Pugh (II) sometimes showed a trace of a halo; the storing had afforded sufficient time for the smaller amount of alkali, or whatever substance produces the turbidity, to diffuse. The formation of the halo in the case of type I is therefore rather a difference of degree than of kind.

Two strains, both foreign ones, diverge considerably from the others. We will deal first with Sp 38. This is a Danish strain received in the early part of this year (1923) from the State Serum Institute, Copenhagen. It is said to be the most common pathogenic type in Denmark, and is a representative of Wulff's (1921) type A. It is also said not to agglutinate with any of the Gordon type sera. I found, however, that it is partly agglutinated by a type II serum when the latter is employed at × 20 titre strength. In its length of life on trypagar it conforms to type I (see Table II). The colony on thick trypagar plates is yellow like type II, but numerous papillæ are sometimes, but not always formed. A slight halo is produced. The other strain, 46 B is an American strain, and is one used by Wadsworth (1920). It agglutinates well with type II serum at titre strength but it agrees with Sp 38 in its relatively short life on trypagar. It also forms a yellow pigment, and papillæ frequently occur on the surface of the colony. There is a slight halo. One mark of distinction is that the colony is always decidedly smaller than those of other strains I have worked with. These two strains seem to possess several features in common, and 46 B appears definitely to belong to Gordon's type II even if there is some doubt about Sp 38. More than one author has expressed the view that type II represents a complex group. Thus Wadsworth, Gilbert and Hutton (*loc. cit.*) say: "It seems possible that type II is a complex group, and that its members cannot be properly differentiated by the agglutination reaction, although strains which react typically may be classified in it." Tulloch (1920) found type II to be a more complex group than the others, and advises that two different strains should always be used in doing the absorption test. It is thus clear that strains are met with which, although apparently falling into one or other group, do not entirely conform to type, and as time goes on it is only reasonable to suppose that more intermediate forms will be discovered. Since Gordon proposed his four types several authors have reported strains which fail to agglutinate with any of the type agglutinating sera. Thus Fildes (1920) states: "Even among epidemic cocci (Naval), strains may occur commonly which do not belong distinctly to any of Gordon's types, but fall between them."

There seems no doubt that we shall have to continue to classify meningococci in the future, and perhaps such cultural tests as those described in this paper may supplement agglutination tests. Enough evidence has been accumulated to show that the latter test leaves much to be desired in the case of meningococcus.

CONCLUSIONS.

(1) By cultivating meningococci on plates composed of a thick layer of trypsin-broth-pea-agar, differences are obtained in the appearance of the colonies which correspond remarkably well with the broad division of meningococci into two main serological groups.

(2) Viability tests on the same medium yield a somewhat similar grouping, except that strains of type I are sharply marked off from all the others including type III, by their short life on this medium.

(3) These features may be usefully employed to replace or supplement serological differentiation.

REFERENCES.

Arkwright, J. A.—(1909) "Varieties of the Meningococcus, with Special Reference to a Comparison of Strains from Epidemic and Sporadic Sources," *J. Hyg.*, **9**, 104.

Fildes, P.—(1920) "The Serological Classification of Meningococci," *Brit. J. Exper. Pathol.*, **1**, 44.

Gordon, M. H., and Murray, E. G.—(1915) "Identification of the Meningococcus," *J. R. Army Med. Corps*, **25**, 411.

Griffith, F.—(1916) *Rep. Local Govt. Board*, New Series, No. 110, p. 41.

Martin, W. Blair. M.—(1910) "The Isolation of the Gonococcus and its Differentiation from Allied Organisms," *J. Path. & Bact.*, **15**, 76.

Nicolle, M., Debains, E., and Jouan, C.—(1918) "Études sur les Méningocoques et les Sérums Antiméningococciques," *Ann. Inst. Pasteur*, **32**, 150.

Osborne, T. B.—(1909) 'The Vegetable Proteins.'

Tulloch, W. J.—(1920) *Med. Res. Council. Spec. Rep. Ser.*, No. 50, p. 113.

Wadsworth, A. B.—(1920) "Comparison of the Potency of Polyvalent Antimeningococcus Serum Produced with Four and Six Representative Strains and that Produced with Sixty Strains, as Determined by the Agglutination Titre," *J. Exp. Med.*, **33**, 107.

Wadsworth, A. B., Gilbert R., and Hutton, A.—(1920) "Study of the Classification of Meningococci," *ibid.*, **33**, 99.

Wulff, F.—(1921) "Étude comparative sur les Méningocoques, types anglais et danois," *Compt. rend. Soc. de Biol.*, **85**, 620.

EXPLANATION OF PLATES.

Fig. 1.—Strain S.A. (I) on thick trypagar. Note the papillæ and halo.

Fig. 2.—Alternate colonies of (1) strain Pugh (II) and (2) strain C.6 (III) on thick trypagar.

Fig. 3.—Alternate colonies of (1) strain Pugh (II) and (2) strain 15 (III) on thick trypagar.

Fig. 4.—Alternate colonies of (1) strain W.30 B. (I) and (2) strain Pugh (II) on thick trypagar. Crowded.

Fig. 5.—Strains on thick glucose agar. 1 = strain S.A. (I); 2 = strain Pugh (II); 3 = strain Chase (III); 4 = strain Taplow (IV).

Fig. 6.—Strains on thick ordinary agar. Numbers as in Fig. 5.

Fig. 7.—Strains on thick trypagar containing phenol red. Numbers as in Fig. 5.

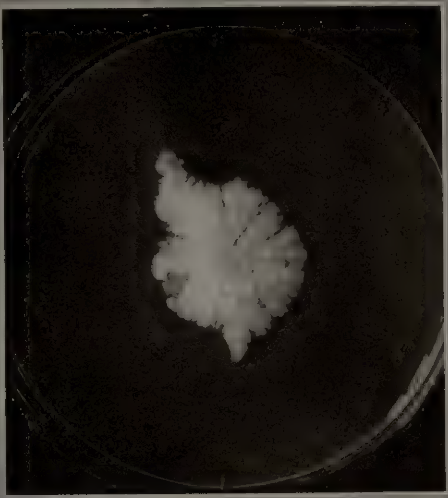


FIG. 1.

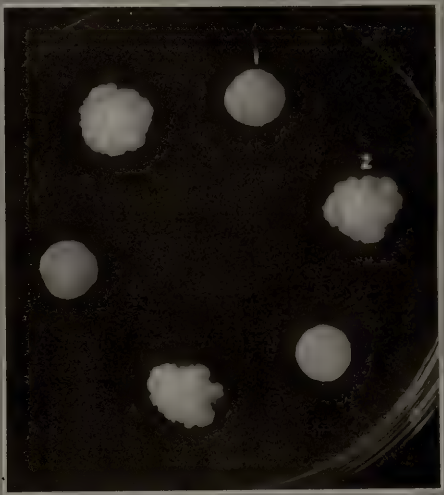
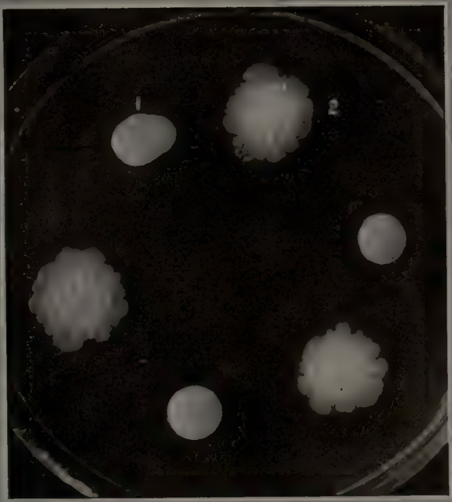


FIG. 2.



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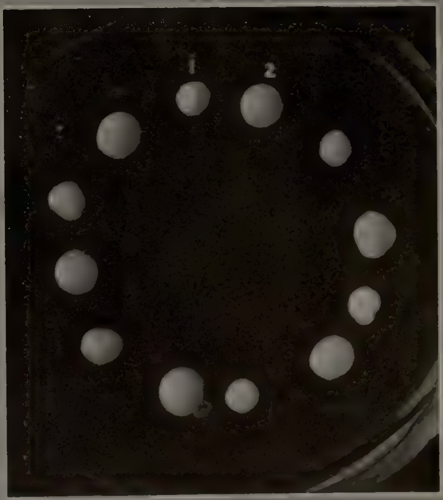
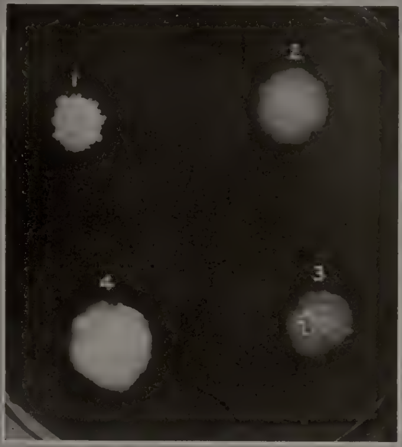


FIG. 4.

Atkin. Plate I.



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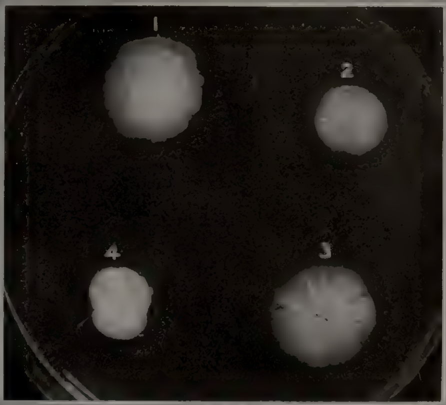

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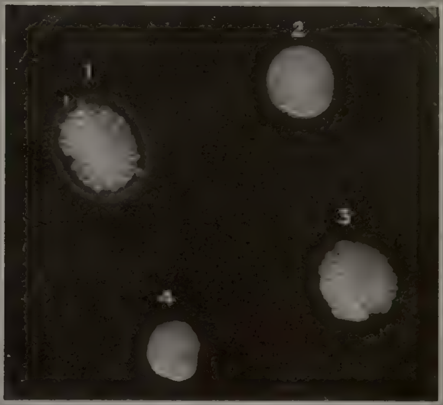

FIG. 7.

Atkin. Plate II.

THE THYROID GLAND IN EXPERIMENTAL RICKETS.

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That definite changes take place in the thyroid gland in certain pathological conditions has been repeatedly demonstrated. Simpson (1906) was one of the first to describe changes in some acute diseases, while Marine and Lenhart (1909) published a report on the examination of 75 thyroids from various conditions and noted marked alteration in the microscopical appearances. Farrant (1913) found that hyperplasia similar to that seen in exophthalmic goitre occurred in such diseases as infantile diarrhœa, measles, diphtheria and whooping-cough, and could be produced experimentally by the injection into animals of diphtheria toxin. Later the same author (1914) reported on the examination of 700 thyroids from as many different conditions as he could obtain them. He found that some diseases were associated with acute or complete hyperplasia of the thyroid, some with chronic or colloid hyperplasia, while others were associated with no change from the normal. Lane (1919) states that in chronic intestinal stasis the thyroid is first hypertrophied and later becomes atrophied. McCarrison (1914) in his work on experimental goitre found that keeping rats in dirty, infected cages produced an acute hyperplastic condition of the thyroid. He obtained the same appearances after subcutaneous injection of bacterial and other toxins. He has recently (1923) confirmed the former observation in pigeons, and found that the hyperplasia could be prevented by scrupulous cleanliness and to a less extent by administration of chlorine water. He states also that cod-liver oil affords complete protection against goitre produced by insanitary conditions.

A paper has just been published by Sorour (1923) giving the results of his observations on the effects of various diets which had been supposed to cause rickets in rats, and he includes a description of the thyroids. He states that the condition was not produced. He describes in some of the thyroids, particularly in those taken from rats which had been kept in darkness, a hypertrophy and microscopical appearances similar to those seen in Basedow's disease.

DESCRIPTION OF EXPERIMENTS.

The following experiments were carried out by Noël Paton and Watson in this laboratory in an investigation into the ætiology of rickets, and most of them have already been published (Paton, Findlay and Watson, 1918; Paton and Watson, 1921, 1923). Prof. Noël Paton examined the thyroids from the first experiment and noted changes in their histological appearances, which he communicated to the Physiological Society at a meeting in Edinburgh in

1920. These seemed to indicate that a routine examination of the thyroid throughout the subsequent experiments might prove of interest. Prof. Noël Paton has kindly allowed me to use the material for this, and I have examined the thyroid of as many of the animals as possible—in all, 43 pups.

Experiment.	Dog.	Thyroid.	Condition of pup.	Remarks.
I	D2	Normal	Normal	Kept in country.
	D3	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B1	Normal	Normal	Kept in country.
	B2	Commencing hyperplasia	,,	Kept in country for fortnight, then in laboratory for 3 weeks.
	B3	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B4	,, ,, (slight)	,,	Running free in laboratory.
	B5	Acute hyperplasia	,,	Confined in cage in laboratory.
II	D1	Normal	Normal	Kept in country.
	D2	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B1	,, ,,	,,	,, ,, ,,
	B2	Normal	Normal	Kept in country.
	B4	Acute hyperplasia	Rickets	Confined in cage in laboratory.
	B6	,, ,, (slight)	,,	Running free in laboratory.
	B7	ditto	,,	,, ,, ,,
VII	1	Normal	Normal	—
	2	,,	,,	—
	3	,,	Slight rickets	—
	4	,,	Normal	—
	5	,,	,,	—
	7	,,	,,	—
VIII	2	,,	,,	—
	3	,,	,,	—
	4	,,	,,	—
	5	,,	,,	—
	6	,,	,,	—
XVI	1	,,	,,	—
XVII	5	,,	Rickets (healing)	Diet included olive oil.
	6	,,	Rickets (healed)	Diet included olive oil, then cod-liver oil.
	8	,,	Normal	Diet included olive oil.
XVIII	1	Chronic hyperplasia	,,	—
	2	,, ,,	Rickets from Ca-starvation	Ca-deficient diet.
	3	,, ,,	Normal	—
	4	,, ,,	Rickets from Ca-starvation	Ca-deficient diet.
XIX	1	Normal	Rickets	Subdued light. Cod-liver oil after rickets developed.
	4	,,	Normal	Subdued light.
XX	1	Chronic hyperplasia	Rickets	—
	2	,, ,,	,,	Olive oil added to diet.
	3	Acute hyperplasia	,,	,, ,, ,,
XXI	1	,, ,,	,,	Ca-free diet.
	2	,, ,,	,,	Calcium lactate added to diet.
	3	,, ,,	,,	Calcium sulphate added to diet.
	4	,, ,,	,,	Ca-free diet.
	5	,, ,,	,,	Calcium carbonate added to diet.

Full details of these experiments as regards the development or non-development of rickets may be found in the papers referred to above, so a very short *résumé* here will suffice. Experiments I and II had for their object the demonstration of the effect of confinement in the laboratory in contrast to controls of the same litter kept in the country. The latter were normal, while all the others developed rickets, although those pups which were allowed to run free in the laboratory were affected in a milder degree than those confined in cages.

Experiments VII and VIII were performed to discover what effect a diet poor in milk fat had on the occurrence of rickets. Only one of this series showed any sign of the condition, and that of a very mild degree. Dog XVI, 1 was one of a series of eight controls for Experiments XVII and XIX, being of the same litters. The former experiment was an investigation into the part played by olive oil in the production of the disease, while the latter was to find the effect of subdued light. In this series three of the pups whose thyroids I examined developed rickets. Two of these received cod-liver oil and recovered completely, while the other improved spontaneously to such an extent that the histological report on the costo-chondral junctions was given as "? early rickets." Experiment XX was a repetition of Experiment XVII, but here all the pups showed signs of rickets, although pups 1 and 2 remained healthy till almost the end of the experiment, while pup 3 developed the condition early, and was so ill that it had to be killed three weeks before the others.

Experiments XVIII and XXI were to investigate the effect of calcium starvation. The two pups in Experiment XVIII on a Ca-deficient diet developed a rachitic condition, which, however, should perhaps not be looked upon as true rickets. In accordance with Korenchevsky's suggestion (1922) I have noted this condition in the table as "rickets from Ca-starvation." In Experiment XXI all the pups became very rickety, although three of them were receiving calcium salts added to their diet. Their condition may be regarded as true experimental rickets which abundance of Ca was unable to prevent.

DESCRIPTION OF THE THYROIDS.

In the accompanying table the condition of the thyroids and the results of the experiments are given. It will be noted that the thyroids fall into three groups—normal, acute hyperplasia, and chronic hyperplasia. I have adopted these terms from Farrant's paper already quoted, as the appearances he describes under these names agree so closely with those in the pups of the above experiments.

Acute or complete hyperplasia (Fig. 2).—Such vesicles as are evident are empty of colloid, and very irregular in outline. Between these is a mass of cells, partly formed of collapsed vesicles hardly recognisable as such, and partly by proliferated cells. The vesicles are lined by cells definitely columnar in shape. The vascularity is greater than normal. Fig. 1 is from a normal pup.

Some thyroids which undoubtedly fall into this group present appearances less advanced (Fig. 3). These are marked "slight" in the table, and it is noteworthy that they are all taken from pups of Experiments I and II which were running free in the laboratory, and which, although showing signs of rickets, were affected to a less degree than those confined.

Chronic or colloid hyperplasia (Fig. 4).—The vesicles are well filled with colloid but have irregular shapes. The walls are often made up of two or three layers of cells instead of the normal single row. There are numerous invaginations of the wall into the lumen of the vesicle, the cells lining which, however, being cubical. In general appearances this type resembles the acute, with the main difference that the vesicles are filled with well-stained colloid.

Pups 1 and 2 of Experiment XX showed this condition. They had only very recently developed rickets, while No. 3, which had suffered from the disease for some time, showed acute hyperplasia. All the pups in Experiment XVIII had similar thyroids, although none had developed *true* rickets as mentioned above. It seems possible that they had been affected in a fashion similar to, but milder than, those which developed acute hyperplasia, and it is conceivable that they might have become rachitic had they been allowed to live longer.

DISCUSSION OF RESULTS.

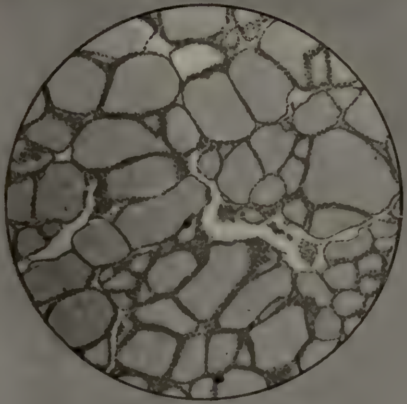
A cursory examination of the table shows that of the 43 pups under review every case of rickets was associated with acute hyperplasia of the thyroid. In Experiments I, II and XXI the changes in that gland were very marked, but, as pointed out above, varied with the severity of the rickets. In Experiments VII and VIII, where only one very mild case of rickets occurred, the thyroids were normal.

Quite definite rickets developed in the pups of Experiments XVII and XIX, but they all improved before they were killed, especially those which got cod-liver oil, which recovered completely. It is interesting to note that the thyroids in these cases were normal. That cod-liver oil has a beneficial action on rickets has long been an accepted fact, but its curative effect on experimental goitre has only recently been demonstrated by McCarrison. In these experiments the cod-liver oil definitely improved the rickets, and presumably, as all pups with acute rickets had hyperplastic thyroids, it restored that gland to normal.

I have found that cod-liver oil has a marked influence on the colloid content of the thyroid vesicles. Rats of the same litter were kept under exactly similar conditions and fed on a carbohydrate diet. Half of the rats received cod-liver oil in addition. The thyroids of the latter were made up of vesicles well filled with colloid (Fig. 5), while those getting no oil had glands with small empty vesicles (Fig. 6). Butter-fat was tried in place of cod-liver oil but was found to have no effect, the thyroids of the animals receiving it and those of the controls having small empty vesicles as in the controls of the other experiment. This condition is similar to that described as hyperplasia and is apparently prevented by the administration of cod-liver oil.

Mellanby (1921) has found similar appearances in the thyroids of animals fed by him on various fats, but he interprets them in a different way. He found that animals fed on a diet containing cod-liver oil had normal thyroids, while those getting butter-fat showed hyperplasia. He considers the butter-fat as the causal agent of the hyperplasia, but if my suggestion is right it would appear that the true cause was probably infective or toxic in nature, from which the others were protected by the cod-liver oil.

Considering how closely these changes in the thyroid in acute experimental



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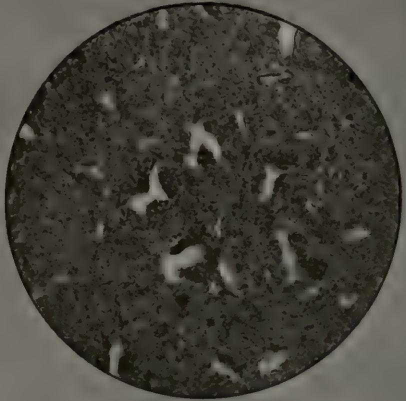

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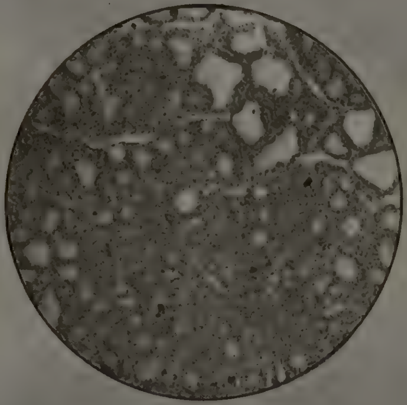

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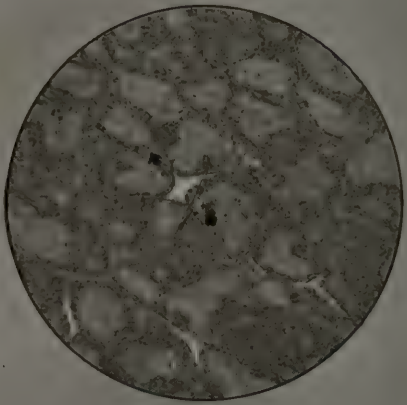
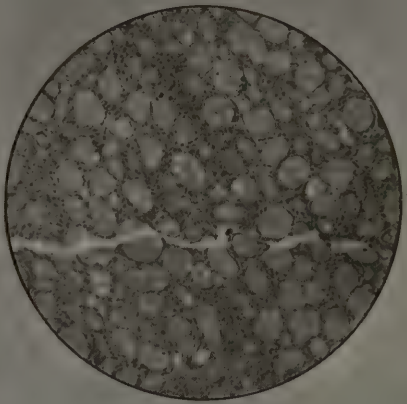


FIG. 4.



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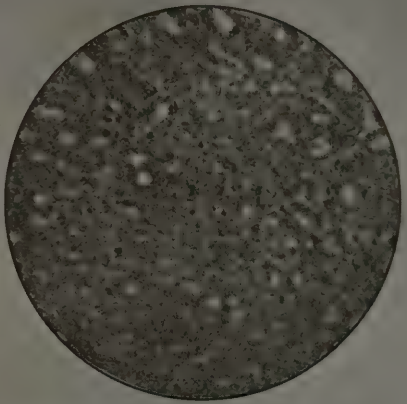

FIG. 6.

Murray.

rickets agree with those described by Farrant in various diseases, it seems possible that here, too, they are the result of a toxæmia. That such a state should occur in these pups which developed rickets is not inconceivable when one considers the experiments carefully. Whatever the apparent cause of the disease, be it confinement, poor nutrition, or subdued light, it is obvious that the adverse conditions to which the animal was subjected would be most likely to favour the development of some infection. In Experiment XX one of the pups was suffering from some such condition—so much so in fact that it had to be killed before the others of the litter—and it was in this animal that most marked rickets and thyroid changes were observed.

There has been a considerable amount of work done by various observers in an attempt to prove the "infective theory" of rickets, but so far it has not been conclusive. It is interesting to find in the examination of these thyroids what might be called presumptive evidence in favour of this theory.

CONCLUSIONS.

1. With the occurrence of rickets in pups there are changes in the thyroid resembling those seen in some infections.
2. These observations show that the rachitic-like changes of Ca-deficiency are not associated with the changes in the thyroid which occur in what may be called true rickets.
3. True rickets may attack pups on a Ca-rich diet as shown by Experiment XXI.
4. Cod-liver oil acts on the thyroid in some way either by preventing destruction or increasing production of colloid.

My sincere thanks are due to Prof. Noël Paton for kindly help during the prosecution of the work, and to Mr. Bell, who prepared the photographs.

REFERENCES.

Farrant, R.—(1913) *Proc. R. Soc. Med.*, **6**, Sect. of Path., 21.—(1914) *Ibid.*, **7**, 49.

Korenchevsky, V.—(1922) *Med. Res. Council. Spec. Rep. Ser.* No. 71, London. (H.M. Stationery Office.)

Lane, W. A.—(1919) 'The Operative Treatment of Chronic Intestinal Stasis,' London. (Oxford Press.)

McCarrison, R.—(1914) *Ind. J. Med. Res.*, **1**, 505.—(1923) *Ibid.*, **11**, 1.

Marine, D., and Lenhart, C. H.—(1909) *Arch. Int. Med.*, **4**, 440.

Mellanby, E.—(1921) *J. Physiol.*, **55**, p.vii.

Paton, D. Noël, and Watson, A.—(1921) *Brit. J. Exper. Path.*, **2**, 75.—(1923) *Ibid.*, **4**, 177.

Paton, D. Noël, Findlay, L., and Watson, A.—(1918) *Brit. Med. J.*, **2**, 625.

Simpson, J. W.—(1906) *Scot. Med. & Surg. J.*, **19**, 504.

Sorour, M. F.—(1923) *Beitr. z. path. Anat. u. z. allg. Path.*, **71**, 467.

DESCRIPTION OF FIGURES IN PLATE.

Fig. 1.—Dog's thyroid, normal.
Fig. 2.—Dog's thyroid, showing acute hyperplasia.
Fig. 3.—Dog's thyroid, showing acute hyperplasia of a milder degree.
Fig. 4.—Dog's thyroid, showing chronic hyperplasia.
Fig. 5.—Thyroid of rat which had cod-liver oil added to its diet.
Fig. 6.—Thyroid of control rat receiving no fat of any kind.

VARIATIONS IN THE DISTRIBUTION OF CO_2, CHLORINE AND CALCIUM IN THE CELLS AND PLASMA OF THE BLOOD IN TETANY.

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THAT there exists a relationship between tissue irritability and anion concentration in the blood has been demonstrated by several investigators (MacCallum, Loeb, Mathews and Tisdall). That the tissue irritability which is indicative of a condition of tetany is related in some way to the degree of ionisation of calcium has also been determined experimentally (Cruickshank, 1923[1]).

In a recent paper (1923[2]) the author has shown that there are two distinctive phases in the development of tetany, one a non-toxic phase, in which the associated blood condition is one of alkalosis, the other a distinctly toxic condition, in which acidosis is marked. That these two phases may play a part in determining the degree of ionisation of the divalent salts is a possibility worth bearing in mind. Freudenberg and György maintained in 1913 that the free calcium in the blood was in equilibration with the bound calcium in the tissues, and suggested that tetany was due to the fact that a loss from the blood of free calcium resulted in a withdrawal of calcium from muscle and nerve tissue. In 1920 they demonstrated that an increase in the pH of the blood would result in an increase in combined calcium, and in the same year Collip (1920) showed that in alkaline solution calcium salts are less ionised than they are in acid solutions. It would seem, therefore, a possibility that the condition of acute tetany is one in which the pH is lowered, accompanied by a decided loss of calcium from the tissues; and further, it would appear that the condition is due not so much to a loss of calcium in the blood bathing the tissues, though that does obtain, but rather to a loss of combined calcium, which renders the tissues more prone to the toxic effects of the irritant monovalent salts.

But while experimental evidence is brought forward to show variations in the amounts of ionic and colloidal salts in the blood, these variations should at present be regarded as concomitant changes due to a causative factor rather than being *per se* the cause of the acute condition following thyroparathyroidectomy in animals.

MacCallum (1920) and his co-workers have brought forward evidence that in tetany produced by pyloric obstruction there is an increase in the CO_2-combining power of the plasma and a decrease in its chloride content.

Hastings, Murray and Murray (1921) corroborate this, with the additional note that the calcium content of the plasma is slightly increased. Tisdall (1922[2]), who has made a more detailed investigation of gastric tetany in adults, found that the calcium content was normal, namely 10·6 mg. per cent., the chlorides had decreased from 360 mg. to 215 mg., the sodium had fallen from 353 mg. to 287 mg., while the CO_2-combining power had risen from 65 to 103 vols. per cent. It is apparent, then, that the CO_2-combining power of the blood and the sodium : calcium ratio are disturbed in the opposite direction in gastric tetany to that which obtains in parathyroid and other types of tetany. In parathyroid tetany the usual results have been a fall in calcium from a general average of 10·0 mg. to that of 6·0 mg. per cent. in whole blood, and a quite definite reduction in the sodium and in the CO_2-combining power of the blood, these changes being accompanied by a lowering of the pH of the blood.

It is known that in the maintenance of normal function the ratio of the inorganic ions of the blood is of far more importance than are changes in the absolute amounts of each. The part played by inorganic salts in causing infantile tetany has been investigated by Kramer, Tisdall and Howland (1921); they found that calcium was markedly diminished, while the phosphates and magnesium remained practically constant; they make no reference, however, to chlorides.

The nature of the alterations in the inorganic salts of the blood in parathyroid tetany has been more fully studied by Gross and Underhill (1922). It should be noted, however, that the estimations made are of the variations in the elements as such, and not of the variations in the amounts of these elements present in the ionic as opposed to the colloidal state. Such variations can be accurately determined by the method of compensation dialysis as has been done for calcium (Cruickshank, 1923[1]). The average values for the inorganic elements in normal animals estimated by Gross and Underhill in milligrammes per 100 grammes by weight of whole blood are Cl 310·0, total P 36·0, Ca 5·6, K 30·0, Na 310, Mg 5·5. For tetany animals they obtained results which show a marked change in certain of the inorganic elements of the blood, namely, a fall in chlorides of 30 mg. and in calcium of 4·0 mg. per 100 gm. of whole blood, while the sodium and magnesium content remained constant or only fell slightly and the phosphorus and potassium increased by an average figure of 10·0 and 5·0 mg. respectively. The important point here is not those absolute amounts but the variation in certain ratios.

That calcium varies directly with the H-ion concentration in physicochemical mixtures of calcium and carbonate was first suggested by Rona and Takahashi (1913). If the H-ion concentration of the mixture remains constant then the Ca-ions and HCO_3-ions will vary in inverse proportion, or if the H-ion concentration increases, then the Ca-ion will increase in proportion to the diminution of the carbonate in accordance with the equation,

$$\frac{[\mathrm{Ca}] \times [\mathrm{HCO_3}]}{[\mathrm{H}]} = \mathrm{K}.$$

In 1915 Loeb found that the ratio of the sum of the monovalent elements to the sum of the divalent elements must be maintained constant in order to preserve normal irritability of nerves. Calculation from the figures of Gross and Underhill of the ratio of monovalent to divalent elements indicates that such a ratio approaches a constant figure. The average figure for the ratio of $\frac{(Na) + (K)}{(Ca) + (Mg)}$ is 31·25; other ratios that are of importance are that of Na : Ca and K : Ca. Tisdall regards the former of importance, while Gross and Underhill seem to regard the latter with that of P : Ca of greater significance. They have shown that in parathyroid tetany the $\frac{(Na) + (K)}{(Ca) + (Mg)}$ ratio changes from the average figure of 31·5 to one of 46·0, while the ratio of K : Ca changes from an average of 5·1 to one of 18·5. Knowing that sodium and potassium salts act as irritants to nervous tissue, and that any increase of these in the blood-stream will precipitate or aggravate an attack of tetany, and that the action of the divalent salts is diametrically the opposite, one can understand the possible importance of a knowledge of these ratios in the treatment of the disease. While such ratios are of interest and value, what is of greater value is a knowledge of the degree to which the colloidal condition of the various elements has been disturbed, and some indication as to alterations in the distribution of these elements.

As to the former, namely, alterations in the ionic and colloidal state of the elements, evidence has been brought forward with regard to calcium which shows that there is an almost complete breakdown of colloidal calcium in acute tetany—an event which is in agreement with those *in vitro* experiments which show that any increase in H-ion concentration of solutions of calcium salts is associated with a liberation of Ca-ions. As to whether or not the cell membrane has suffered damage and thereby become more permeable to ions, or whether the cellular function has been disturbed so that ions are lost, must be investigated both by dialysis and a determination of the distribution of these ions in the cells and in the plasma.

With regard to the chlorides, it has been indicated that there may or may not be a definite fall in the chlorides of the whole blood. It will be seen from Table I that during the first stage of tetany there is usually no marked alteration in the chloride content of the plasma and corpuscles, but that, with the development of acute tetany, the animals showing all the signs of a severe toxic condition, there is a loss of chloride from the cells, and further that the loss increases up to some 40 per cent. of the total cell chloride. It has also been shown that this loss may run almost parallel with the diminution in the alkali reserve of the blood, which may amount to some 60 per cent. of the normal. The CO_2-combining power of the blood is lowered, but the most marked loss of CO_2 is that which occurs from the cells. These results would seem to indicate that the tetany toxin is of the nature of a non-volatile acid, which depletes the alkali reserve and attacks the hæmoglobin molecule, reducing its base and thus liberating Cl-ions, which, however, do not increase the chlorine cellular content. The loss of chlorine from the cell is so marked, amounting from 10 to 40 per cent. of its total chloride content, that it seems justifiable to assume that the tetany poison has damaged to a considerable extent the corpuscular economy, thereby liberating Cl-ions from their colloidal combination.

TABLE I.

	CO₂.				Chlorides.						
	Blood.	Plasma.	Cells.	Cell percent. of total	Blood.	Plasma.	Cells.	Cell percent. of total.	Hæmatocrit.	pH.	Remarks.
1*a*	43·912	33·085	10·872	24·6	·625	·450	·175	28·0	3·5	7·40	Normal.
b	48·110	35·59	12·52	26·5	·615	·438	·177	28·8	3·2	7·45	T. mild.
c	26·298	21·515	4·653	17·9	·670	·514	·156	23·3	3·5	7·25	T. acute.
	40·8	34·5	57·4	—	8·8	(14·0+)	18·5	—	—	—	Percent. loss.
2*a*	46·65	33·82	12·53	26·8	·505	·375	·130	25·70	4·0	7·35	Normal.
b	42·355	32·780	9·575	20·1	·540	·424	·116	21·50	3·6	7·40	T. mild.
c	35·964	28·718	7·186	20·0	·510	·391	·119	32·30	3·7	7·30	T. acute.
	22·9	15·5	42·7	—	0·99	(4·27+)	8·46	—	—	—	Percent. loss.
3*a*	40·836	29·77	11·066	27·10	·650	·482	·168	25·86	3·2	7·35	Normal.
b	43·884	38·197	5·687	12·96	·595	·504	·091	18·85	2·35	7·30	T. definite.
c	39·606	34·00	5·606	14·15	·620	·528	·092	17·90	2·0	7·25	T. acute.
	3·0	—	49·3	—	4·6	(9·5+)	39·3	—	—	—	Percent. loss.
4*a*	42·50	32·45	10·04	23·5	·615	·462	·153	24·9	3·0	7·35	Normal.
b	40·098	32·890	7·208	18·0	·570	·448	·122	21·4	3·0	7·45	T. definite.
c	38·560	30·366	8·194	21·2	·535	·444	·091	17·0	2·95	7·35	T. acute.
	90·7	3·36	18·45	—	13·00	3·9	40·60	—	—	—	Percent. loss.

TABLE II.

	Per cent. in whole blood.	Per cent. in plasma.	Plasma per cent. of whole.	Per cent. in cell.	Cell per cent. of whole.	Remarks.
Calcium	9·12 mg	8·11 mg.	88·9	1·01 mg.	11·1	Normal.
	5·72 ,,	5·26 ,,	91·9	0·46 ,,	8·1	Acute tetany.
	37·40	35·20	—	54·4	—	Per cent. change.
Chlorine	·599 gm.	·442 gm.	73·9	·156 gm.	26·1	Normal.
	·563 ,,	·449 ,,	79·8	·114 ,,	20·2	Acute tetany.
	6·1	±	—	26·91	—	Per cent. change.
CO_2	43·45 vols.	32·25 vols.	74·20	11·20 vols.	25·8	Normal.
	35·1 ,,	28·60 ,,	81·5	6·50 ,,	18·5	Acute tetany.
	19·20	11·31	—	41·9	—	Per cent. change.

Changes in:				
$[CO_2]$.	$[BHCO_3]$.	Alk. reserve.	pH.	Remarks.
18·39	17·25	17·25	7·37	Normal.
13·05	12·26	5·26	7·31	Acute tetany.
29·00	29·40	30·18	8·15	Per cent. change.

TABLE III.—*Equilibration Data from Blood of Normal Dog.*

Atmosphere.		Blood.						
CO_2 per cent.	pCO_2.	CO_2 per cent.	$[CO_2]$.	$[H_2CO_3]$.	$[BHCO_3]$.	R.	pH.	
1·92	13·44	24·45	10·82	·4032	10·42	25·90	7·593	—
3·97	27·79	34·56	15·43	·8337	14·60	17·54	7·424	—
5·81	40·67	39·90	18·00	1·2201	16·78	13·75	7·318	—
10·23	71·61	49·31	22·00	2·1485	19·85	9·26	7·147	—

Equilibration Data from Blood of Tetany Dog.

CO_2 per cent.	pCO_2	CO_2 per cent.	$[CO_2]$.	$[H_2CO_3]$.	$[BHCO_3]$.	R.	pH.	
2·68	18·90	18·78	8·39	·5670	7·83	14·06	7·327	—
3·43	24·20	21·47	9·58	·7260	8·86	12·20	7·266	—
6·50	45·80	29·50	13·17	1·3740	11·80	8·60	7·114	—
11·30	79·75	39·30	17·56	2·3925	15·17	6·35	6·980	—
3·904	(27·33)	35·95	16·05	(0·820)	(15·13)	15·22	(7·39)	Normal.
3·595	(95·33)	22·30	9·96	(0·760)	(9·20)	12·02	(7·26)	Tetany.
—	—	—	6·80	—	6·42	—	7·39	At normal pH.

Change in alkali reserve—8·71 mm. $BHCO_3$.

Note.—Figures in brackets are obtained by interpolation on the curve obtained from equilibration data from the blood of normal and tetany dogs. $R = \frac{BHCO_3}{H_2CO_3}$. $pH = \log R + pK_1$. $pK_1 = 6·18$.

In Table II are given the average results of experiments made to determine the distribution of calcium, chlorine and CO_2 in the plasma and cells and alterations in the bicarbonate content and alkali reserve of the whole blood of normal and tetany dogs. The $[BHCO_3]$ content of the blood was determined from equilibration data as shown in Table III. To determine accurately the changes in alkali reserve it is necessary to extrapolate the $[BHCO_3]$ value of the tetany blood to that which would obtain for tetany blood under a CO_2 tension which would restore the normal pH. The average figures given in Table II show that in acute tetany, so far as the blood is concerned, the alterations in calcium, chlorine and CO_2 are associated with some disturbance of intracellular function. That such a loss in alkali reserve as is shown here should bear a relationship to the loss of chlorine from the blood-cells while the chlorine content of the plasma remains almost stationary would indicate a definite alteration in the manner of the combination of alkali and oxygen by the hæmoglobin molecule. To determine the nature of the damage within the cell one would require to know what changes are occasioned in the ionic and colloidal contents of the cellular elements, and for this a method for the estimation of these forms in the corpuscle is still wanting.

CONCLUSIONS.

In the early stages of tetany there is usually no pronounced change in the chloride content of either corpuscles or plasma. In later stages, and particularly when toxic symptoms appear, there is a reduction of chloride, especially in the corpuscles, which may lose from 10 to 40 per cent. of their chloride. Accompanying and probably directly related to this is a fall in the alkali reserve, particularly of the corpuscles, and a reduction of the calcium content of both corpuscles and plasma. It is suggested that the toxic agent in tetany is a fixed acid.

REFERENCES.

Collip, J. B.—(1920) *Amer. J. Physiol.*, **52**, 483.
Cruickshank, E. W. H.—(1923)[1] *Biochem. J.*, **17**, 13.—(1923)[2] (in the press).
Freudenberg, E., and György, Z.—(1920) *Biochem. Ztschr.*, **110**, 299.—(1921) *Jahrb. f. Kinderh.*, **96**, 5.
Gross, E. G., and Underhill, F. P.—(1922) *J. Biol. Chem.*, **54**, 105.
Hastings, A. B., Murray, C. D., and Murray, H. H.—(1921) *Ibid.*, **46**, 223.
Kramer, B., Tisdall, F. F., and Howland, J.—(1921) *Amer. J. Physiol.*, **20**, 560.
Loeb, J.—(1902) *Arch. f. d. ges. Physiol.*, **91**, 248.—(1915) *J. Biol. Chem.*, **22**, 423.
MacCallum, W. G., Lintz, J., Vermilye, H. N., Leggett, J. H., and Boas, E.—(1920) *Bull. Johns Hopkins Hosp.*, **31**, 1.
Mathews, A. P.—(1904) *Amer. Journ. Physiol.*, **11**, 455.
Rona, P., and Takahashi, D.—(1913) *Biochem. Ztschr.*, **49**, 370.
Tisdall, F. F.—(1922)[1] *J. Biol. Chem.*, **54**, p. 35.—(1922)[2] *Ibid.*, **50**, 329.

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